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ACTIVE SITE AND KINETIC
STUDIES ON CARBONIC ANHYDRASE III

by

JENNIFER B SHELTON BSc

A thesis submitted in partial fulfilment of the requirements
of the Council for National Academic Awards
for the degree of Doctor of Philosophy

Research conducted at Sheffield City Polytechnic
in collaboration with the Department of Human Genetics,
University of Michigan Medical School, Ann Arbor, Michigan, USA

April 1991

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I would like to thank Ms Pat Rose for her meticulous and accurate typing of this thesis.

Finally, my thanks are extended to my family for their constant support and encouragement, and their magnanimous donation of study space.

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2 July 1988

Conference on "The Cellular Physiology and Molecular Genetics of the Carbonic Anhydrases", University College, London.

3 19 - 23 March, 1990

Symposium on "The Function of Carbonic Anhydrase - its Role in Ion and Gas Transfer", Annual Meeting of the Society of Experimental Biology, Warwick University.

4 June 1988

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5 July 1990

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4 January 1989 - June 1989

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ABSTRACT

Active Site and Kinetic Studies on Carbonic Anhydrase III

Jennifer B Shelton

Carbonic anhydrase III (CA III) was purified to homogeneity from red skeletal muscle of both chicken and human. Analysis of purification procedures revealed that preparations may be easily contaminated with a protein possessing phosphoesterase activity. This could be the source of acid phosphatase activity previously attributed to CA III.

The effects of various buffers, anions and phosphorylated metabolites on the activity of these isoenzymes towards bicarbonate and several ester substrates were examined. Phosphate (P_i) enhanced the HCO_3^- dehydration activity of chicken and human CA III, as measured by the pH-stat assay system. Pipes, mops and hepes buffers had no effect.

The K_M of chicken CA III appeared to decrease with P_i , whereas k_{cat} remained constant. Exposure of chicken CA III to high $[P_i]$ followed by low $[P_i]$ resulted in retention of P_i -enhanced activity for up to 20 minutes. This slow dissociation could thus sustain the P_i -effect under conditions of fluctuating $[P_i]$. This response was pH-dependent between pH 6.5-7.5.

Pyrophosphate, HSO_3^- , ATP, ADP, PEP, 1,3-BPG and 3-PG each enhanced bicarbonate dehydration activity and activation by one species precluded further activation by P_i . No phosphatase activity by CA III was evident.

Activation of CA III by the arginine-modifying reagent, 2,3-butanedione (BD), was also investigated. A comparison of this activation with that of phosphate, for the HCO_3^- dehydration reaction, suggested common features.

K_M and k_{cat} were determined for 4-nitrophenyl acetate hydrolysis by chicken CA III. BD-modification increased k_{cat} , but had no effect on K_M , whilst P_i was without effect. This may substantiate the premise that HCO_3^- dehydration and esterase sites are spatially separated on CA III.

The physiological implications of these findings are discussed.

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ABBREVIATIONS USED IN THE TEXT

| | |
|-----------|--|
| Å | Ångstrom unit |
| ADP | adenosine-5'-diphosphate |
| Arg | arginine |
| AT | <u>anterior tibialis</u> |
| AR | AnalaR |
| ATP | adenosine-5'-triphosphate |
| BD | 2,3-butanedione |
| 1,3-BPG | 1,3-bisphosphoglyceric acid |
| BSA | bovine serum albumin |
| BTB | bromothymol blue |
| c-AMP | cyclic adenosine monophosphate |
| CA | carbonic anhydrase |
| Co | company |
| CoCA | cobalt carbonic anhydrase |
| CSF | cerebrospinal fluid |
| conc | concentration |
| D | dextro |
| DISC-PAGE | discontinuous polyacrylamide gel electrophoresis |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| DNSA | dansylamide |
| EC | Enzyme Commission |
| EDTA | ethylenediaminetetra-acetic acid |
| EEO | electroendoosmosis |
| EDL | <u>extensor digitorum longus</u> |

| | |
|-----------|---|
| Fig | figure |
| FG | fast glycolytic |
| FOG | fast oxidative glycolytic |
| g | gramme |
| GPR | general purpose reagent |
| h | hour |
| Hb | haemoglobin |
| hepes | 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid |
| Hg | mercury |
| HNTAS | 2-hydroxy-5-nitro- -toluenesulphonic acid sultone |
| kd | dissociation constant |
| kD | kilodalton |
| k_{cat} | catalytic constant |
| K_I | inhibition constant |
| K_M | Michaelis constant |
| l | litre |
| L | laevo |
| Lys | lysine |
| M | molar |
| min | minute |
| mops | 4-morpholinepropane sulphonic acid |
| ms | milliseconds |
| NMR | nuclear magnetic resonance |
| No | number |
| 2-NPA | 2-nitrophenyl acetate |

| | |
|------------------|--|
| 4-NPA | 4-nitrophenyl acetate |
| °C | centigrade |
| OAA | oxaloacetic acid |
| PCr | phosphocreatine |
| pI _E | isoelectric point |
| P _i | inorganic phosphate |
| Phe | phenylalanine |
| pipes | 1,4-piperazinebis(ethanesulphonic acid) |
| RIA | radioimmunoassay |
| RMM | Relative Molecular Mass |
| mRNA | messenger ribonucleic acid |
| rpm | revolutions per minute |
| s | second |
| SD | standard deviation |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SE | standard error |
| SO | slow oxidative |
| SR | sarcoplasmic reticulum |
| TEMED | N,N,N',N'-tetramethyl ethylene diamine |
| VA | <u>vastus</u> |
| vol | volume |
| V _{max} | maximum initial velocity of an enzyme catalysed reaction |
| v/v | volume to volume ratio |
| w/v | weight to volume ratio |

Carbonic anhydrase (Carbonate dehydratase EC 4.2.1.1), is a ubiquitous enzyme which rapidly catalyses the physiologically important hydration of carbon dioxide or reversely the dehydration of bicarbonate. This zinc metalloenzyme also acts as a general acid-base catalyst towards a number of 'synthetic' substrates, such as 2- and 4-nitrophenyl acetate and toluene based sultones. The enzyme is known to exist in several forms, with carbonic anhydrase (CA III) being the soluble form recently characterised from red skeletal muscle. The function of CA III in muscle is obscure, although it appears to be exclusively located in predominantly red slow-oxidative muscle fibres under precise hormonal and neuronal control, and in concentrations approaching 4-10% of the cytosolic protein.

CA III has strikingly different properties compared to the other more fully characterised isoenzymes CA I and CA II, namely it possesses a lower activity, has a lower sensitivity to sulphonamide inhibition, is sensitive to anion inhibition and is reported to exhibit an in vitro acid phosphatase activity. Examination of the active site residues unique to CA III reveal the presence of three basic residues at positions 64 (Arg/Lys), 67 (Arg) and 91 (Arg). Basic residues are normally located in the active sites of enzymes which bind phosphorylated substrates or modulators. Consequently, establishing a link between CA III and phosphate or phosphorylated metabolites could be of fundamental importance is elucidating the role of this isoenzyme in muscle physiology and the energy metabolism of the muscle cell.

Results presented in this thesis suggest that there is a link between phosphate and CA III. With respect to bicarbonate dehydration

activity, this isoenzyme is responsive to inorganic phosphate, several phosphorylated metabolites and some anions. The response appears to be an ionic interaction resulting in a transiently stable enzyme-phosphate complex. Preliminary data indicate that there is some involvement of arginine residues. An extension of the study to the esterase activities of CA III suggests that inorganic phosphate has little effect on these catalytic activities.

1.1 HISTORICAL BACKGROUND

The carbonic anhydrases are a group of enzymes that catalyse the reaction, $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$. The enzyme was given the name carbonic anhydrase by Meldrum and Roughton in 1932, when they discovered that CO_2 escaped from haemolysed blood more quickly than could be accounted for by the uncatalysed conversion of bicarbonate to carbon dioxide. A year later they published a method for the purification of the enzyme (Meldrum and Roughton, 1933), and they demonstrated its inhibition by anions, namely cyanide, azide and sulphide. Their work was independently confirmed by Stadie and O'Brien (1933).

Keilin and Mann (1940) were to discover several years later that carbonic anhydrase is a zinc containing enzyme, with one zinc-bound ion per molecule. This was the first clearly defined physiological function for this metal ion. These authors demonstrated the inhibitory effect of unsubstituted sulphonamide and they suggested that the sulphonamide was bound in some way to the metal ion; a fact that has been verified in more recent years by X-ray diffraction and spectroscopy (Lindskog, 1969).

It was some twenty years before attention was again focused on the carbonic anhydrases with the improved purification techniques of Lindskog (1960). It led to the discovery that red blood cells contain two distinct carbonic anhydrases that differ in catalytic and other properties (Nyman and Lindskog, 1964; Rickli et al 1964). These enzymes were designated B and C on the basis of their electrophoretic mobility on starch gels. The C form has a higher activity towards CO_2 hydration than the B form, with a turnover approaching $2 \times 10^6 \text{ s}^{-1}$.

A system based on Roman numerals was introduced by Rickli and Edsall (1962) with the high activity C form reclassified as CA II and the low activity form B as CA I. With both nomenclatures in operation, the addition of newly isolated isoenzymes and secondary polymeric forms, led to a confusing situation. The quandary was clarified by the international recognition of the numerical system (Tashian and Carter, 1976) and any secondary alterations of the primary products of these genes are designated CA I (+ 1), CA I (+ 2) etc.

Thus, the third genetically distinct isoenzyme isolated in 1976 from mammalian muscle was designated CA III (Holmes, 1976; Register et al, 1978). The membrane-bound form found in mammalian liver and kidney was classified CA IV (Whitney and Briggie, 1982; Wistrand, 1984) and in 1984 a fifth form isolated from the mitochondria of hepatocytes of mammalian liver was designated CA V (Dodgson et al, 1984). More recently a sixth secretory enzyme (CA VI) has been discovered (Fernley et al, 1984; Murakami and Sly, 1987) and also a genetically distinct CA VII locus (Montgomery, 1988).

The carbonic anhydrases comprise a multigene family with seven known isoenzymes to date. CA I and CA II have been more fully investigated than the type III isoenzymes. A study of any one member of the enzyme family cannot be undertaken without considering the other members; consequently the characteristics of CA III are not only discussed per se, but in relation to the other isoenzymes, particularly CA I and CA II. Thus in subsequent sections, information pertaining to isoenzymes other than CA III is included where relevant.

1.2 GENERAL DISTRIBUTION OF THE CARBONIC ANHYDRASE ISOENZYMES

As predicted from the fundamental importance of the reaction catalysed by CA, the distribution of this enzyme is widespread in the animal and plant kingdom and in some micro-organisms (Carter, 1972). The distribution of the various isoenzymes has been most fully investigated in mammalian species, but some comprehensive studies have been undertaken in non-mammalian and plant groups (Holmes, 1977; Graham et al, 1984).

CA II is generally considered to be the most widely distributed isoenzyme and it has been isolated in many different tissues and cells including erythrocytes, kidney, liver, lungs and the retina (Maren, 1967). Recent published data indicates that the membrane form, CA IV, may also be expressed in a wide range of human cells (Carter et al, 1990; Sapirstein et al, 1983; Wistrand, 1984). The distribution of CA I and CA III, however, is very much more restricted and expression is primarily confined to erythrocytes and red skeletal muscle respectively.

The mitochondrial form, CA V, has been identified in guinea-pig and rat liver parenchymal cells and recently in the inner mitochondrial membrane of rat kidney cortical cells (Dodgson and Contino, 1988). The secretory type VI isoenzyme is found in the salivary glands of humans (Murakami and Sly, 1987) and ovine parotid glands (Fernley et al, 1984) and has been identified in the salivary gland of the rat (Feldstein and Silverman, 1984).

1.3 GENETIC ASPECTS AND COMPARATIVE IMMUNOCHEMICAL STUDIES

In terrestrial vertebrates, the CA system comprises at least seven genes (Tashian, 1989). Human genes CA I, II and III are closely linked on the long arm of chromosome 8 and they may in fact, all be linked at a single complex locus (Wade et al, 1986). The gene duplication events that gave rise to this multiple CA gene cluster, since they are represented by both the bird and mammalian group, must have occurred prior to the divergence of reptiles and mammals about 300 million years ago. Strong evolutionary pressure must have been necessary to retain the linkage of these three isoenzymes.

Human CA VI and CA VII are located on chromosomes 1 and 16 respectively. The structures of CA genes from plants and lower organisms have yet to be characterised.

With the exception of CA I, human isoenzymes all appear to have seven exons, with six introns at approximately the same positions and they range in size from 9.8 kb to 17 kb. Quantification of transcript messenger from human CA III indicates that the mRNA is 1.8 kb long, but only half of the transcript encodes protein. It is, however, one of the most abundant classes of clones in the human muscle cDNA library, with a frequency of message even higher than actin mRNA (Wade et al, 1986).

Comparisons of complete and partial sequences from CA I, II and III from a particular species usually show sequence homologies of more than 50%. Despite this degree of structural similarity, there is virtually no immunological cross-reactivity between these three isoenzymes, indicating that they have essentially different immunological domains (Nishita and Matsushita, 1988).

There is a high degree of sequence homology if a particular isoenzyme, eg CA I, is compared to the same isoenzyme of a related species. Thus, a comparison of the amino acid sequence of bovine CA III (Tashian et al, 1980) and equine CA III (Wendorff et al, 1985) reveals 95% homology. Not surprisingly, common antigenic determinants can be demonstrated between horse, cow, rat, dog and cat CA III.

As more carbonic anhydrases are discovered and characterised, fewer amino acids unique to a particular form are apparent, they nevertheless share many conserved residues. Not only can these comparisons aid in the construction of phylogenetic trees, but they assist in relating catalytic properties to the unique structure of the particular isoenzyme. The remainder of this introduction will focus on muscle CA III, its unique properties and structure and the relevance of the work undertaken. It is essential though, for the reasons stated, to be constantly aware of the wider context of the multigene background and where necessary information will be drawn from this extensive pool.

1.4 LOCALISATION AND REGULATION OF CA III IN MUSCLE

There is a complex distribution of carbonic anhydrase isoenzymes in the neuromuscular system and isoenzyme content is dependent both on the type of muscle and the age and sex of the animal (Jeffery et al, 1988, 1990). Moreover, there is evidence that isoenzyme expression is linked to the hormonal and neuronal status of the tissue.

Carbonic anhydrase III is by far the major soluble CA in red mammalian skeletal muscle and primarily accounts for any CA activity in the cytoplasmic fraction of this tissue (Frémont et al, 1987). Expression of CA III is mainly confined to striated muscle, since only trace amounts are evident in the smooth muscle cells of the human uterus and in the myoepithelial cells of the mammary and prostate glands (Vaananen and Autio-Harmainen, 1987). Extensive immunohistochemical study of human cardiac muscle indicates the absence of CA III in this tissue, a useful distribution with regard to utilising CA III as a skeletal muscle marker. The standard muscle marker, creatine kinase, is prevalent in all muscle systems and thus provides a less sensitive indicator of skeletal neuromuscular disorders than CA III. Early trials suggest that CA III readily permeates the muscle membrane, particularly after exercise, in patients with myogenic and neurogenic disorders (Vaananen et al, 1988). Thus, raised serum CA III levels might prove a useful diagnostic tool in the future.

Immunohistochemical examination of muscle sections and activity studies of the particulate fraction of red skeletal muscle suggest that the soluble CA III co-exists with two membrane-bound isoenzymes (Geers et al, 1985; Bruns et al, 1986). These carbonic anhydrases associated with the sarcoplasmic reticulum and sarcolemma are

uncharacterised with regard to isoenzyme type, yet they probably play an important role in the regulation of the tissue (Deutsch, 1987).

Vertebrate striated muscle can be classified into three main types, although transitional forms occur. Type 1 fibres represent the ancestral form and have the potential for sustained contraction; a property facilitated by the plentiful supply of mitochondria, a good blood supply and a high myoglobin content. The array of enzymes in these slow twitch fibres is representative of an energy supply maintained by oxidative phosphorylation. The other two types of muscle fibre, IIa and IIb, have acquired the capacity for anaerobic metabolism. The difference between IIa and IIb fibres is that IIa fibres additionally obtain their energy by oxidative phosphorylation. Thus, I, IIa and IIb fibres are sometimes referred to as SO (slow oxidative), FOG (fast oxidative glycolytic) and FG (fast glycolytic). In reality, most muscles are a mixture of fibre type, which are in a state of constant flux depending on the muscular activity of the individual; and type IIa fibres probably represent an intermediate stage of ongoing fibre transformation.

The skeletal muscles of rat, however, provide an ideal experimental system for studying the location and activity of fibre specific proteins; since the soleus comprises 87% of type I fibres, the tibialis anterior 66% IIa fibres and the superficial vastus lateralis 97% type IIb fibres (Armstrong and Phelps, 1984; Ariano *et al*, 1973). The gastrocnemius and extensor digitorum longus also have a high proportion of IIb fibres.

Interestingly, a comparison of the CA III content and activity of the different rat muscles described above suggests that CA III is linked specifically with slow twitch type I fibres (Riley *et al*, 1982; Lönnerholm, 1984; Jeffery *et al*, 1986). Virtually no CA III is

present in muscles containing primarily IIb fibres.

Whether or not CA III is expressed in muscles containing IIa fibres is equivocal, with conflicting reports, an acceptable situation given that these muscles can contain an undefined proportion of type I fibres and may be in a transitional state.

CA III is randomly distributed within the sarcomere with no evidence of localisation in the mitochondria, nuclei, T-tubules or any specific band structure of the striated muscle (Frémont et al, 1988¹).

The specific distribution of CA III described in rats is essentially similar in other mammals (Nishita and Matsushita, 1987; Frémont et al, 1988²).

The development of the specific fibre types and hence muscle differentiation depends to some extent on the pattern of neuronal stimulation of that particular muscle. Characteristic neuronal activity appears to be necessary for the synthesis of several fibre specific isoforms, for example, the synthesis of adult fast and slow myosin heavy chains (Whalen et al, 1981). Denervation of the neonatal rat soleus muscle prevents the emergence of type I fibres. In addition, electrostimulation of anterior tibialis (AT) muscle in a slow pattern switches on the production of slow muscle proteins. Consequently, stimulation of AT muscle in a slow pattern results in the production of CA III four days after neuronal activation, and levels are maintained for 42 days postelectrical stimulation.

Denervation of the rat fast muscles, extensor digitorum longus (EDL) and AT results in striking changes in the CA III level 15 days post-denervation (Carter et al, 1988). The CA III activity as measured by RIA and mRNA levels, increases dramatically even though the EDL and AT muscles contain mainly type II fibres. It is

postulated that control is exerted after transcription and that constant positive control is necessary, which on removal or alteration causes a reversion to the type I fibre form. The increase in CA III level evident in aging rats during puberty corresponds to the establishment of specific fibre/nerve impulse patterns.

Thyroidectomy mirrors the effects of denervation and three months post thyroidectomy an eight-fold increase of CA III activity is evident in EDL muscle, concomitant with an increase in type I fibres, a slight increase in type IIa fibres and a decrease in type IIb fibres.

The exact molecular signal for switching on muscle CA III synthesis is unknown and appears related to either hormonal control, a growth factor secreted by nerve endings, a neurotransmitter, or a combination of one or more of these factors; but it is directly related to fibre type composition and hence to differentiation and development. Examination of CA III DNA sequence indicates that the promoter region associated with transcription of human CA III is not characteristic of a tissue specific gene (Edwards *et al*, 1988). The gene may, however, possess an upstream enhancer containing a possible thyroid hormone receptor and/or glucocorticoid receptor binding element (Edwards, 1990). Research is underway to determine which regions are transcriptionally active.

Notwithstanding the complexity of independent regulation, there is some evidence that regulation of one isoenzyme may to some extent be controlled by the status of its relatives.

Reference has been made to the close spatial arrangement of the CA I, II and III genes. The interrelationship of CA III and CA II in the erythrocyte system was investigated in a Belgian family carrying a CA II deficiency gene. The asymptomatic heterozygous parents and

heterozygous son had the normal range of erythrocyte CA III, but the clinically affected CA II deficient son had a CA III level elevated by approximately 100% (Carter et al, 1984). A similar correlation between the gene products of CA I and CA II in the pig-tailed macaque monkey has also been demonstrated (DeSimone et al, 1973).

1.5 LOCALISATION AND REGULATION OF CA III IN RAT LIVER

In the rat, unlike many other mammals, CA III is also found in the liver, localised in the hepatocytes (Laurila *et al*, 1989). It exhibits sexual dimorphism with concentrations of CA III in the male rat liver 10-20 times those found in the female (Carter *et al*, 1984). Using RIA, Carter demonstrated the androgen linked control of CA III gene expression in rat liver. Testosterone administration to ovariectomised females induced a 4-5 fold increase in liver CA III. Conversely, castration and estradiol treatment produced a 75-90% reduction in mature male liver CA III.

The expression of an androgen controlled protein may be influenced by more than one hormone. Rat α -2 globulin for instance, is controlled by testosterone, thyroxine and growth hormone. There is some evidence that thyroxine regulates CA I and CA II in some species; and patients suffering from hyperthyroidism and hypothyroidism have a correspondingly decreased or increased level of red blood CA I, and to a lesser extent CA II. A study of growth hormone response in male and female rats indicates that CA III may also be controlled by a multiplicity of hormones. Though not direct evidence for involvement of growth hormone, several studies have demonstrated a negative relationship between the mean body weight of mammalian species and the level of red blood cell CA activity (Magid, 1967; Dodgson and Foster, 1983).

1.6 PHYSIOLOGICAL ROLES OF THE CARBONIC ANHYDRASES

The only known physiological function of carbonic anhydrase is to catalyse the reversible hydration of CO_2 . In view of the fundamental importance of this reaction, it is not surprising that the enzyme plays a major part in many metabolic and physiological processes. These functional roles, excluding muscle function which is dealt with in a separate section, are briefly described.

The role of the carbonic anhydrases in the kidney and in respiration are particularly well documented and researched. The combined renal function of CA II and membrane-bound CA IV maintains acid-base status by reclamation of bicarbonate in the proximal kidney tubule and subsequent secretion of Na^+ in exchange for H^+ (DuBose, 1984). The cytosolic CA II equilibrates CO_2 from the blood with carbonic acid, HCO_3^- and H^+ . In CA II deficiency, only CA IV functions so that H^+ ions are not available for exchange with Na^+ and HCO_3^- , the filtrate is not rapidly decomposed to CO_2 and H_2O , and the urine becomes progressively alkaline.

In the respiratory system CA II and CA I have two roles. Firstly to maintain pH and facilitate uptake and release of O_2 by haemoglobin and secondly to facilitate rapid CO_2 transport by production and consumption of HCO_3^- .

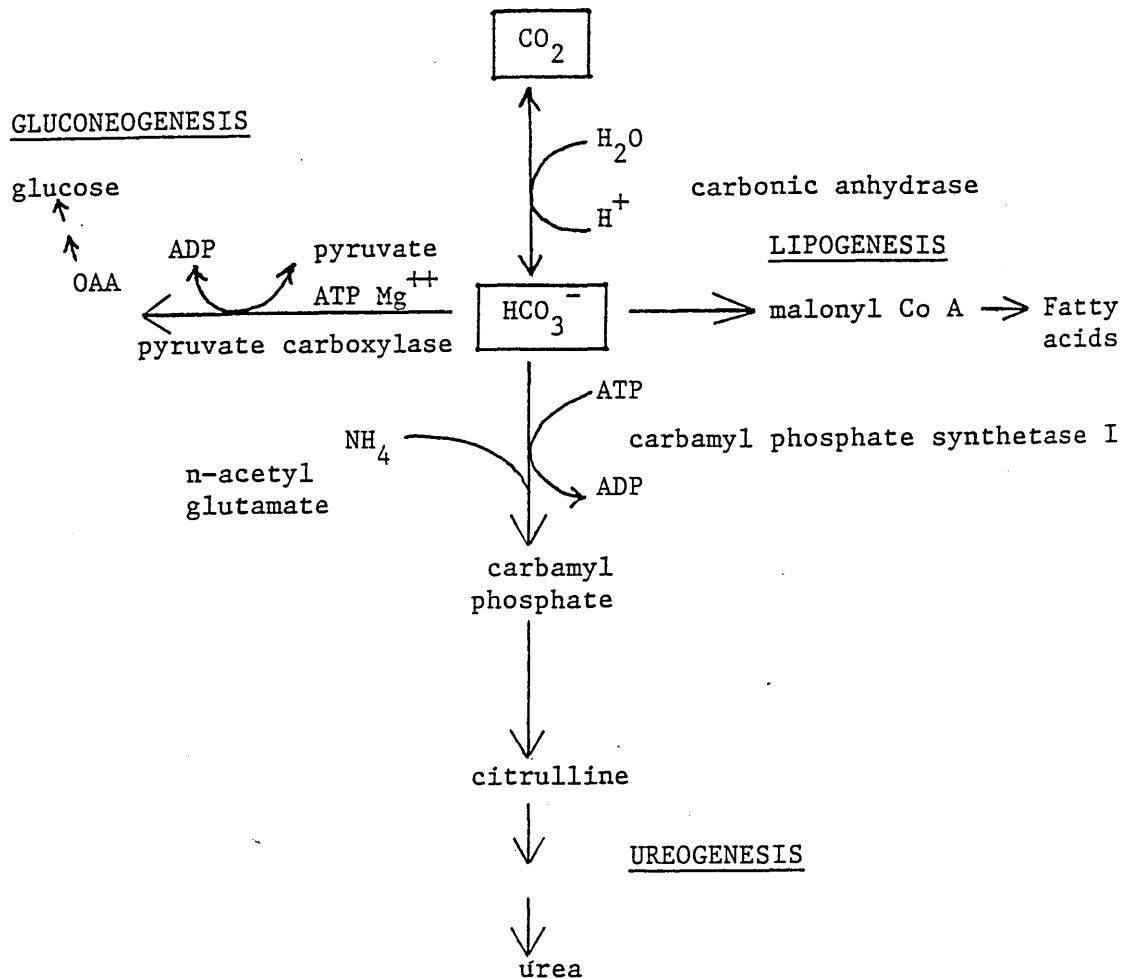
Electrolyte transport mediated by pH changes in the gastro-intestinal tract, the CNS and the ciliary processes are largely controlled by CA (Kishida et al, 1984). Prolonged administration of acetazolamide, an inhibitor of CA, results in a general malaise in patients, 50% of whom experience gastro-intestinal symptoms (Wistrand, 1984). The general malaise is in part probably due to its effect on CAs in the CNS (Sapirstein et al, 1983), and the gastro-intestinal

symptoms due to a combined loss of intestinal function and bicarbonate secreting function of the gastric mucosa. In gastric acid production, the secretory product is not bicarbonate, but hydrogen, a process that is stimulated by gastrin, histamine and cyclic-AMP possibly via a cyclic-AMP dependent protein kinase (Narumi and Kanno, 1973; Narumi and Miyamoto, 1974).

Recent research has implicated CA in a biosynthetic role, in addition to the homeostatic and secretory functions mentioned above. Bicarbonate metabolism is central to the urea cycle, gluconeogenesis and fatty acid synthesis, and the main pathways are depicted in Figure 1.1. Bicarbonate does not readily pass across membranes and it is postulated that mitochondrial CA V provides the necessary HCO_3^- in liver hepatocytes for the first urea cycle enzyme, carbamyl phosphate synthetase (Haussinger and Gerok, 1985; Dodgson et al, 1984). Glucose production in the liver requires bicarbonate for carboxylation of pyruvate, again CA is thought to provide the prerequisite bicarbonate (Metcalf et al, 1985, Dodgson and Foster, 1986).

Studies by Herbert and Coulson (1984) in lizards using ^{14}C -acetate suggest a possible role for a CA in the first carboxylation step in lipogenesis. Recently, Spencer et al (1988) examined the possible involvement of CA in lipogenesis in the fat body of the desert locust. A role for regulation of lipid synthesis in the developing adult is implied (Chegwidden and Spencer, 1990).

FIGURE 1.1 Pathways Involved in Intrahepatocytic HCO_3^-
(Dodgson et al, 1984)



Carbonic anhydrase is also important in the process of shell formation and bone metabolism. Patients deficient in CA II suffer from a distinct form of osteopetrosis and concomitant failure of bone resorption, which results in an accumulation of bone mass (Sly et al, 1983). Parathyroid hormone (PTH) is primarily concerned with the calcaemic response, and CA inhibitors affect the PTH release of calcium from the bone. The enzyme may be activated by PTH in certain bone cells where it aids the resorptive process by the secretion of H^+ . In the chick chorioallantoic membrane, Tuan (1986) concluded

that CA was not part of the active transport of calcium, but that the enzyme has an acidification role promoting accessibility of transport components. Neilson and Frieden (1972) proposed that CA simply provides the requisite bicarbonate for combination with calcium in oyster shell formation in gastropods.

The importance of CA in plants is less well documented. Sixty-seven per cent of the total leaf activity is confined to the chloroplast and thus CA probably plays a vital role in CO₂ fixation or transfer in photosynthesis, or the provision of protons in photophosphorylation (Graham et al, 1984).

1.7 FUNCTIONAL ROLE OF CARBONIC ANHYDRASE III IN MUSCLE

Despite the high level of CA in this tissue and the wealth of knowledge that has accumulated in relation to the roles of CA in other systems described briefly above, the precise function of CA III in muscle is unresolved.

The contracting muscle produces substantial quantities of CO_2 with a corresponding reduction in pH. A model has been proposed for the rapid transport of CO_2 out of the cell whereby muscle CA rapidly hydrates CO_2 to HCO_3^- . The bicarbonate diffuses across the cell to the cell-capillary interphase, where the reverse reaction occurs because the membrane is largely impervious to bicarbonate. Carbon dioxide diffuses a short distance into the blood stream. The H^+ ions are buffered by intracellular phosphate and myoglobin (Gros et al, 1976).

Several in vitro methods for testing this hypothesis have been devised. Gutnecht et al (1977) measured the diffusion of CO_2 across lipid bilayer membranes. In the absence of CA, the diffusion of CO_2 is rate limiting, but in the presence of CA the rate limiting step is the diffusion of HCO_3^- through the unstirred layers. Diffusion of H^+ may become rate limiting, if the solution is poorly buffered. Gros (1976) investigated the flux of CO_2 across millipore filters soaked in phosphate, with and without CA. He found that facilitated diffusion of CO_2 occurred when sufficient CA was present, when a HCO_3^- and CO_2 gradient existed and when the phosphate had a mobility comparable to that of bicarbonate. If phosphate was immobilised by attaching cellulose particles, no CO_2 diffusion was detected, so Gros concluded that CO_2 flux was dependent on a proton transfer system mediated by phosphate buffer.

Can this be equated to whole muscle and if so, which isoenzymes are responsible? Gros (1988) constructed an apparatus to investigate the CO₂ diffusion properties of whole muscle. Carbon dioxide diffusion decreased across the muscle with increasing acetazolamide concentration, the K_i corresponding to that of CA III rather than CA I or CA II.

However, acetazolamide does not readily diffuse into tissue, unlike chlorzolamide or cyanate. Comparative studies using these inhibitors were undertaken on the rat soleus (rich in CA III) and in rat extensor muscle (type II fibres) with respect to isometric twitch, tetanic tension and muscle relaxation time (Geers and Gros, 1988). Both inhibitors caused a decrease in isometric twitch and tetanic tension, but an increase in relaxation time, so the authors concluded that the observed effects were due to inhibition of the sarcoplasmic CA rather than CA III. They postulated that the sarcoplasmic enzyme provides protons for the uptake of calcium essential for continued muscle contraction. This view is supported by Tuan (1986) in the chick chorioallantoic membrane, where CA provides the protons for transport mechanisms, rather than acting directly in calcium sequestration. Isolated sarcoplasmic reticulum from skeletal muscle releases calcium if the pH is abruptly increased from 6.4 to 7.8 and calcium is rebound if the pH is decreased (Nakamaru and Schwartz, 1972).

The distinction between the involvement of CA III or sarcoplasmic reticulum-bound CA often depends on their relative sensitivity towards sulphonamides. Using a high concentration (10⁻³ M) of the sulphonamide, methazolamide, Côté (1989) and his co-workers found that the fatigue properties of rat EDL (type II fibres) were unaffected. Soleus muscle (type I fibres), however,

showed an increased resistance to fatigue. No fatigue effects were observed in the soleus at 10^{-5} M methazolamide. The authors concluded that the observed effects were due to CA III inhibition. At 10^{-5} M methazolamide, the 1/2 relaxation time was increased in the soleus, a property contributed to SR-CA activity.

Assuming that CA III is linked to CO_2 mobilisation and SR carbonic anhydrase with muscle tension and the calcium transport-contraction mechanism, the question still remains, "Why is CA III specifically located in red muscle?" All muscles produce CO_2 . One could argue that CA II with a turnover of 10^6 sec^{-1} could function more efficiently in red skeletal muscle. Moreover, since the CO_2 concentration of muscle is approximately 1 mM, human CA III with a K_M of 40-80 mM (CO_2) would seem an unsuitable isoenzyme in this tissue, unless additional factors were in operation.

Thyroidectomy results in type II to I fibre transformation and a concomitant change in CA III content. Thyroid hormone treatment induces a change in type IIb fibres but no change is seen in type IIa fibres, and yet they have an oxidative potential greater than type I fibres and contain considerably less CA III. Côté (1989) found that type I fibre muscles have five times higher CA III activity than type IIa fibre muscles. If CA III were just concerned with oxidative potential metabolism one would expect it to be present in type IIa fibre muscles in much higher concentrations, under hormonal and/or neuronal control. In type one fibres, oxidation of fatty acids and glycogen are the major metabolic pathways used for ATP production during work of long duration. Fatigue and exhaustion are generally related to a depletion of glycogen stores rather than lipid depletion. However, there is a positive correlation between CA III activity of a muscle and the activity of triacylglycerol lipase

(Miller et al, 1987). CA III may play a role in utilising substrate reserves and preventing the onset of fatigue in slow twitch muscle, that have the capability of sustained work.

It seems an inescapable conclusion that CA III is intimately associated with particular contractile elements but the precise mechanism has so far eluded research. By characterising CA III, and attempting to equate these properties with the physical and chemical characteristics described, it was hoped that the research outlined in this thesis would by some small measure address these interesting problems.

The three-dimensional structures of CA I and CA II, as refined by X-ray crystallography, are broadly similar and a stylised model is shown in Figure 1.2 (Liljas et al, 1972) together with a ribbon model (Figure 1.3, Eriksson et al, 1988). Recent evidence suggests that CA III is structurally similar (Eriksson and Liljas, 1986). The refined structures of the more recently discovered isoenzymes have yet to be determined, although it is clear that some membrane bound CA IV isoenzymes (Whitney and Briggie, 1982; Zhu and Sly, 1990) and the salivary gland CA VI isoenzymes are both glycoproteins (Fernley et al, 1988).

The model depicts a monomeric ellipsoidal protein with the approximate dimensions 41 x 42 x 55 Å, containing approximately 260 amino acids (RMM 29 kD). The overall structure is stabilised by ten chain segments that form a large twisted β -sheet. Six polypeptide chains run almost perpendicular above and below this β -pleated sheet (Nostrand et al, 1979). Interestingly, if the N-terminal and C-terminal ends are pulled apart the protein will form a knot.

FIGURE 1.2 Schematic Representation of the Main Chain Folding in CA I and CA II, Viewing Towards the Active Site

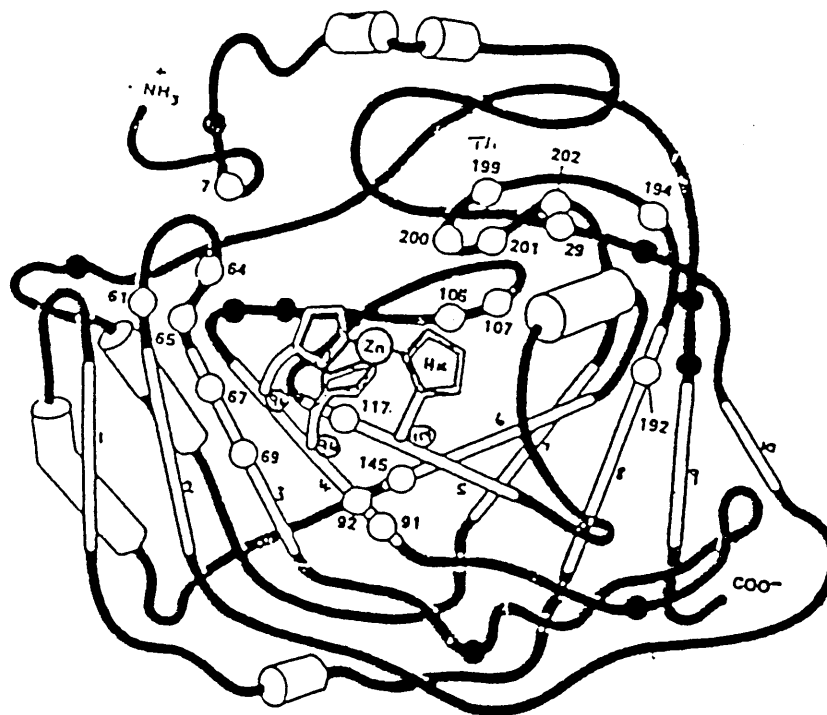
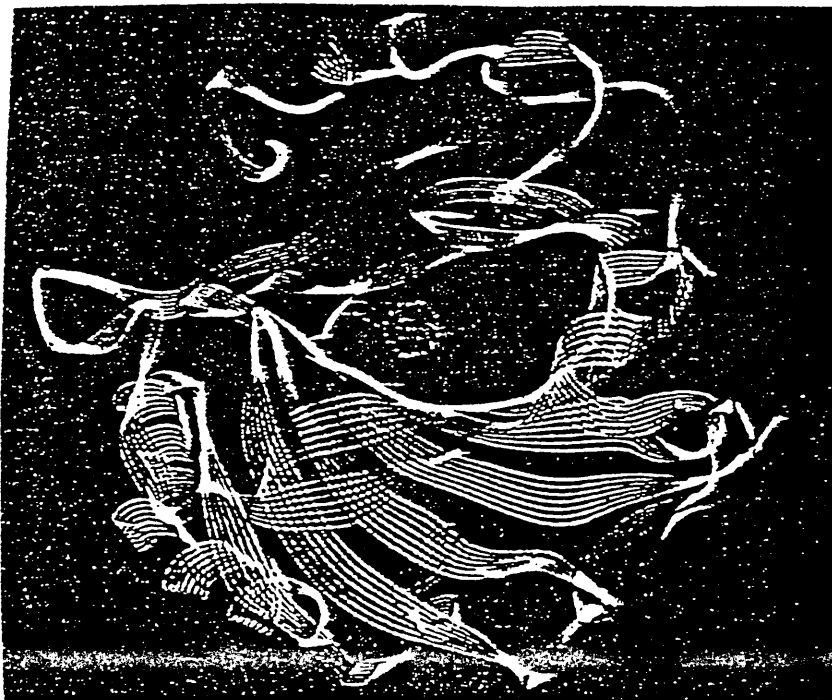


FIGURE 1.3 Ribbon Model of Human CA II



The molecule is stabilised by three aromatic clusters, which are remarkably similar in CA I, II and III. There is, however, one notable difference in cluster A which comprises seven phenylalanine residues in CA I and II, but Phe-66 is replaced by Cys-66 in CA III. A universal feature of CA III isoenzymes is that they contain five cysteine residues, in contrast to CA I and CA II. They are located at positions 66, 183, 188, 203 and 206, and two of these at positions 183 and 188 are reactive to alkylating agents. The muscle isoenzymes thus have a tendency to dimerise, alkylation of these residues prevents dimerisation. The specific effect of dimerisation is unclear and there is no evidence of disulphide bridges in the folding of CA III. The plant carbonic anhydrases have a high cysteine content and characteristically higher relative molecular masses. They appear to consist of subunits of RMM 25-30 kD, each containing a zinc atom and it is reasonable to assume that cysteine plays a role in maintaining the overall structure.

The essential zinc ion, at the base of the conical active site cavity, is situated in the middle of the β -pleated sheet structure between strands 4 and 5, tightly bound to three histidine residues (96, 94 and 119). The fourth or fifth ligand is occupied by a solvent or substrate molecule in an essentially tetrahedral geometry (Lindskog et al, 1983). The mouth of the cavity is bounded by Lys-64 (Arg in chicken), Pro-202, Phe-131 and chain segment 3 (residues 65-71) (Pocker and Sarkannan review, 1978).

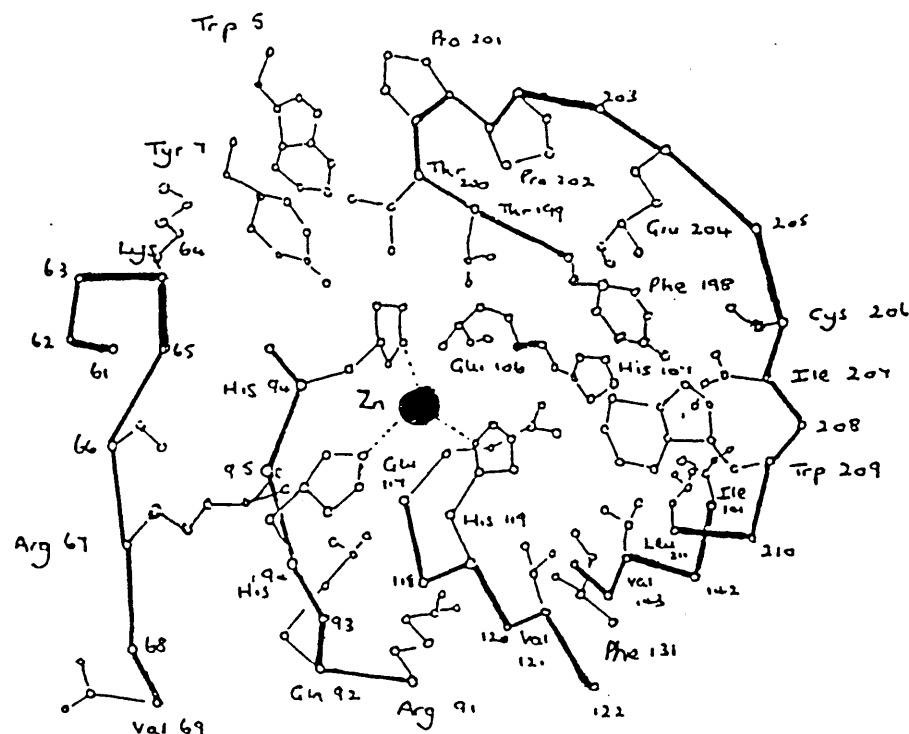
Approximately 30 residues constitute segments 3-6 which form part of the active site and very few conservative replacements have occurred in this area. As more carbonic anhydrases are isolated and sequenced, fewer and fewer residues unique to a particular isoenzyme are apparent; but as the field broadens the amino acid residues common to CA function as a whole can be separated from those that impart specific isoform characteristics. For instance, in all CA I type enzymes examined to date, histidine is present at position 200, which is threonine in CA II and III. Histidine, an invariant residue at position 64 in CA I and CA II is replaced by a basic residue in CA III, generally lysine, but arginine in chicken. CA III always contains two more basic residues at positions 67 and 91. A list of some of the invariant residues "unique" to CA III is shown in Table 1.1.

TABLE 1.1 A Comparison of Active Site Residues in Isoenzymes from Various Species

| | | 'Unique' Active Site Residues | | | | |
|---------------|---------|-------------------------------|-----|-----|-----|-----|
| | | 64 | 65 | 67 | 69 | 91 |
| <u>CA III</u> | Human | Lys | Thr | Arg | Val | Arg |
| | Gorilla | Lys | Thr | Arg | Val | Arg |
| | Ox | Lys | Thr | Arg | Val | Arg |
| | Chicken | Arg | Thr | Arg | Val | Arg |
| <u>CA I</u> | Human | His | Ser | His | Asn | Phe |
| | Monkey | His | Ser | His | Asn | Phe |
| | Ox | His | Ser | His | Asn | Phe |
| | Turtle | His | Ser | His | Asn | His |
| <u>CA II</u> | Human | His | Ala | Asn | Glu | Ile |
| | Monkey | His | Ser | Asn | Glu | Ile |
| | Ox | His | Ser | Asn | Glu | Val |
| | Chicken | His | Ser | Asn | Glu | |

The spatial arrangement of the CA III active site is depicted schematically in Figure 1.4 and is based on the stereoscopic drawings of Notstrand (1979), in conjunction with sequence data of Hewett-Emmett et al (1984). Lysine 64 points away from the active site and may form a salt bridge with Glu-4. Histidine 64 in CA I and CA II has been implicated in the catalytic mechanism, and the substitution of this residue by a basic one in the type III enzyme has often been linked to its low activity (section 1.10). It is postulated that histidine 64 together with some of the adjacent residues forms a "gate" across the active site, which allows a certain degree of rotation and accelerates a proton shuttle mechanism. Arginine 67 is directed towards the zinc moiety and has no other links, but how freely it rotates is not certain (Eriksson, unpublished data). The third basic residue in CA III, arginine 91, points away from the active site and forms a salt bridge with aspartic acid 72 (Eriksson, in press).

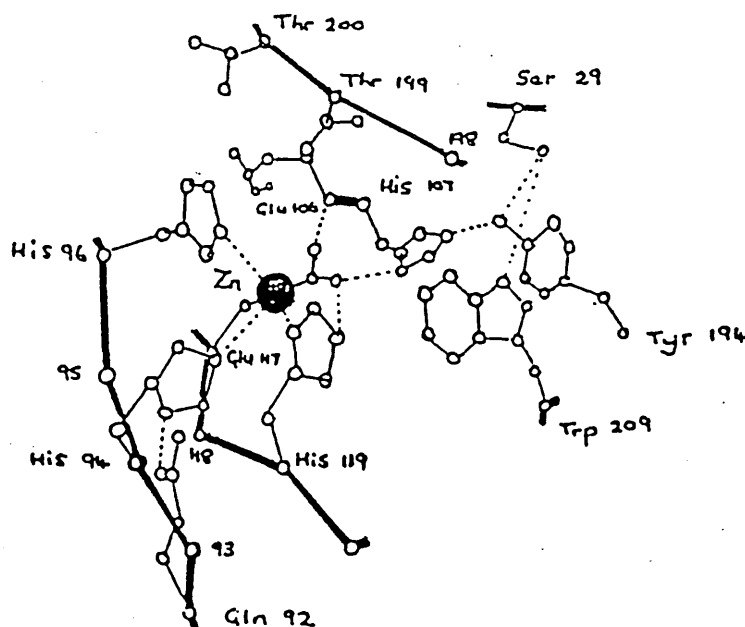
FIGURE 1.4 Active Site Region of Human Carbonic Anhydrase III



Two other invariant residues may be particularly important in determining the 'unique' CA III characteristics. Cys-66 is centrally placed in the active site domain and there is some evidence that treatment of bovine CA III with thiol agents may increase activity (Engberg and Lindskog, 1986). The other residue is phenylalanine at position 198, which is occupied by leucine in CA I and CA II. A network of hydrogen bonds links the zinc bonded $\text{OH}^-/\text{H}_2\text{O}$ and histidines 94, 96 and 119 to a number of other residues, either directly or indirectly (Figure 1.5). This core of residues (Ser-29, Gln-92, Glu-106, His-107, Glu-117, Tyr-194, Thr-199, Trp-209) are always invariant for all the carbonic anhydrases and they consequently play a vital role in the catalytic mechanism (Lindskog, 1983).

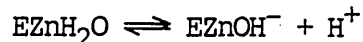
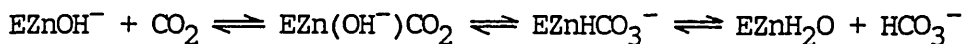
Phenylalanine 198 in CA III is a large aromatic residue with an electron dense cloud which lies near Glu-204 and is likely to affect the hydrogen bonding around Glu-106. It has even been suggested that this large ring may restrict the substrate binding site and effectively narrow the opening of the cavity.

FIGURE 1.5 Hydrogen Bond Network at the Active Site of Carbonic Anhydrase



1.10 CATALYTIC MECHANISM

The precise catalytic mechanism of carbonic anhydrase has been the subject of debate for a number of years despite the simplicity of the reaction and the seemingly straightforward transfer of OH^- from H_2O to CO_2 . One of the difficulties has been to satisfactorily explain the rapid turnover number, which in CA II approaches 10^6 molecules per second (Silverman and Vincent, 1983). It is now generally accepted that there is a direct nucleophilic attack of E-Zn-bound OH^- on CO_2 with the subsequent release of HCO_3^- . Initiation of another round of nucleophilic attack is dependent on a replacement OH^- , which in turn depends on the protolysis of water and the release of a proton. Thus, the rate limiting step is in fact the protolysis of water and a proton transfer step rather than the formation of a new carbon-oxygen bond (Silverman and Lindskog, 1988).



The reaction is fully reversible, and for the hydration of CO_2 by CA II, both k_{cat} and $k_{\text{cat}}/K_{\text{M}}$ have simple pH rate profiles and are dependent on an ionisable group with a pK_{a} near 7.0. A number of proton donors and acceptors have been considered with a pK_{a} of 7.0, the obvious candidate, the zinc bound OH^- itself is discounted because of too low a rate. A shuttle mechanism has been proposed, whereby a proton is transferred via an internal ionisable residue to the surrounding buffer (Khalifah, 1971). Several residues could participate in this role, namely histidine 64 (CA II), one of the

bound imidazole groups or Glu-106. Forsman et al (1988) reported that substitution of histidine 64 by an acidic or basic residue resulted in no change of activity. However, Tu and Silverman (1989) showed that replacing His-64 by Ala-64 decreased the rate of proton transfer by 20-fold, in an unbuffered medium. In addition, the unmodified enzyme is inhibited by micromolar concentrations of Cu^{2+} , whereas the alanine-modified enzyme is unaffected. Evidence suggests, that in CA III, the rate of catalysis is also dependent on a step outside the $\text{CO}_2\text{-HCO}_3^-$ conversion, and it is thus likely that Lys-64 has a significant effect on activity, unless another mechanism is operational (Karlari and Silverman, 1984). The data presented in this thesis indicate that in the muscle isoenzyme, external ions may have a vital role in the conversion of HCO_3^- to CO_2 .

A close examination of cobalt substituted enzymes has shown that in the presence of non-interacting buffers or ion-free solutions, the absorption vs pH profile may not be simply one single dissociating group (Brown, 1987). In vivo, ions like chloride that bind to the metal ion may be important factors in the mechanism.

1.11 KINETIC PROPERTIES OF CA III

In addition to the carbonic anhydrases catalysing the hydration of CO_2 and dehydration of HCO_3^- , they also catalyse many 'synthetic' substrates involving the addition of hydroxide to a carbon-oxygen bond or similar analogue (Magid, 1968). Pocker and Sarkannen (1978) list many of these latter reactions in their excellent review. They include the reversible hydration of aliphatic aldehydes and pyruvic acid, the hydrolysis of carboxyl ester linkages such as 4-nitrophenyl acetate and naphthyl acetate and the hydrolysis of 1-fluoro-2,4-dinitrobenzene (Linkskog *et al*, 1971; Steiner *et al*, 1975). In addition, CA catalyses the hydrolysis of 4-nitrophenyl thioacetate and 2-hydroxy-5-nitro-toluene-sulphonic acid sultone (HNTAS), which involves the breaking of a carbon-sulphur bond and a sulphur-oxygen bond respectively (Sanyal *et al*, 1983).

Carbonic Anhydrase III is largely uncharacterised with regard to many of these 'synthetic' substrates. However, a specific phosphomonoesterase activity has been solely attributed to CA III, leading to speculation that unlike CA I or CA II, the muscle isoenzyme catalyses a reaction involving a phosphorylated metabolite (Koester *et al*, 1981). This theory is partly substantiated by the fact that, despite the similarity in the conserved active site residues of CA I, II and III, they differ markedly in their kinetic parameters. In terms of CO_2 hydration, human CA III has only 3% of the activity of CA II and 20% of the activity of CA I. These percentages do not however, take account of subtle changes in response to ions in the surrounding cytoplasm mentioned in the previous section. The data in this report indicates that these comparative activities may not be absolute values in a physiological situation.

The activity of CA III may also differ with respect to pH dependence. CA I and CA II have sigmoidal pH-rate profiles with points of inflection around 7, attributable to either a single ionisable group or an interaction of two or more acid-base groups (Bertini *et al*, 1987). CA III from bovine (Engberg *et al*, 1985) and feline (Karlari and Silverman, 1984), skeletal muscle and rat liver is independent of pH between 5.5 and 8.0. The pH of muscle varies considerably during strenuous exercise. A study by Bevington (1986) on the internal changes of phosphorylated compounds and pH, during 30 minutes continuous exercise, showed that pH can drop by as much as one unit, from a resting level of 7.03 to 6.25. It has been suggested that in CA III, the pKa is below 5.5 and the zinc bound hydroxide is stabilised by the positive charges in the active site (Silverman and Lindskog, 1988).

The carbonic anhydrases are susceptible to inhibition by monovalent anions and sulphonamides. The anionic 'metal poisons' SH^- , CN^- and NCO^- are the strongest inhibitors with K_i values of 10^{-5} to 10^{-6} M and the progression of activity of the halides is as follows: $\text{F}^- > \text{Cl}^- > \text{Br}^- > \text{I}^- > \text{ClO}_4^-$. There is overwhelming crystallographic evidence that anions such as NCO^- , N_3^- and I^- that inhibit CO_2 hydration bind to the metal ion, either by replacing a coordinated water molecule or by expansion of the coordination sphere.

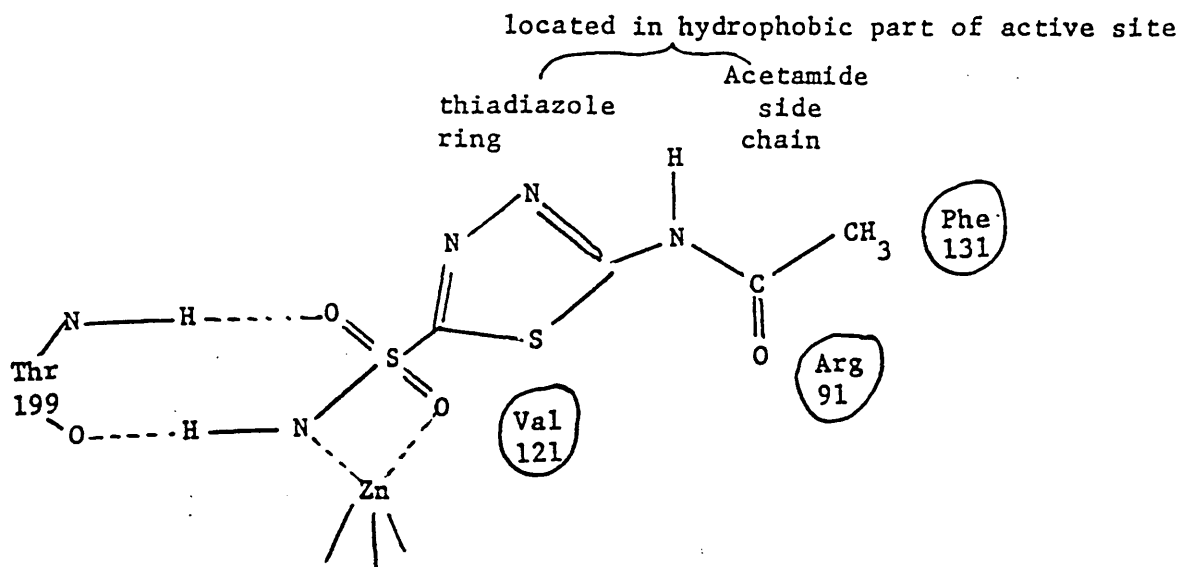
Anion inhibition is pH dependent in CA I and CA II, with strongest binding occurring in the protonated form of the ionising group or groups of the enzyme. As the pH is raised, the inhibition is weakened due to competition with OH^- and HCO_3^- (Maren and Sanyal, 1983; Tibell *et al*, 1984; Pocker and Tanaka, 1978). In feline CA III, the inhibition constant of azide is independent of pH between 6.0-7.5

(CO₂ hydration). Moreover, inhibition by anions is uncompetitive at pH 6.0, but mixed at higher pH values (Karlari and Silverman, 1985).

The susceptibility of each isoenzyme to anion inhibition varies and is often an aid in categorising new isoforms. CA III and CA I are particularly sensitive, whereas CA II is more resistant and the membrane CA IV form, completely resistant to inhibition by halides (Sanyal *et al*, 1982; Maren *et al*, 1980; Shelton and Chegwidan, 1987). In terms of relating molecular structure to characteristic inhibition parameters, there is very little information. In CA I, His-200 affects anion binding particularly in its protonated form when anion inhibition is high. This may account for the low resistance of CA I, but it does not explain why CA III is also sensitive to anion inhibition when it has threonine at position 200, in common with CA II, which has a high resistance.

The interaction of sulphonamides with the carbonic anhydrases has been the subject of intense investigation, because of their clinical importance and their high specificity and potency (Maren, 1984). The R-SO₂NH⁻ complex of the sulphonamide reacts with the zinc moiety and the heterocyclic portion interacts with residues in the hydrophobic part of the enzyme molecule, particularly residues 91, 121 and 131 as shown in Figure 1.6 (Kannan *et al*, 1977).

FIGURE 1.6 Interaction of Acetazolamide with Human CA III



Although many of the characteristics of anion binding are shared by sulphonamide binding, namely competition with respect to OH^- and HCO_3^- , and the pH-rate profiles (Leibman and Greene, 1967), relative resistances of CA I, II and III are different. CA III exhibits greatest resistance and generally CA II is most sensitive. Table 1.2 list the K_i and k_d values for acetazolamide and the corresponding residues at positions 91, 121 and 131. The general resistance of CA III is thought to be due to the invariant basic residue at 91 instead of a hydrophobic acid, as in CA I and CA II. The reduced active site cleft due to the presence of Phe-198 in CA III is probably also a contributing factor. Horse CA I and CA II have identical acetazolamide inhibition constants and interestingly identical residues at positions 121 and 131. Horse CA III is particularly resistant, a property which may be attributed to tyrosine at 131, in addition to the basic residue at 91 (Chegwidden and Shelton, 1987; Chegwidden *et al*, 1986).

TABLE 1.2 Residues Involved in Acetazolamide Binding and the Corresponding Inhibition Constants

| | | Active Site Residues | | | K_i | k_d |
|---------------|---------|----------------------|-----|-----|---------|---------|
| | | 91 | 121 | 131 | μM | μM |
| <u>CA III</u> | Human | Arg | Val | Phe | 50 | 50 |
| | Horse | Arg | Val | Tyr | 1800 | |
| | Chicken | Arg | | | 7 | |
| | | | | | | |
| <u>CA I</u> | Human | Phe | Ala | Leu | 0.2 | 0.1 |
| | Rabbit | Ser | Val | Ile | 0.2* | |
| | Horse | Val | Val | Phe | 0.02 | |
| | | | | | | |
| <u>CA II</u> | Human | Ile | Val | Phe | 0.01 | 0.03 |
| | Rabbit | Ile | Val | Phe | 0.02* | |
| | Horse | Ile | Val | Phe | 0.02 | |
| | Chicken | Val | Val | Phe | 0.03 | |
| | | | | | | |

* CO₂ hydration

K_i = inhibition constant (Venta et al, 1987; Chegwiddden and Shelton, 1987; Sanyal et al, 1982)

k_d = dissociation constant (Osborne and Tashian, 1983)

In CA I and CA II, the 4-NPA esterase activity has many features in common with the CO₂ hydration activity, ie similar pH-rate profiles, anion and sulphonamide inhibition, leading to the conclusion that the hydratase and esterase sites are one and the same (Verpoorte et al, 1967). Recent evidence suggests that in CA III the two reactions show no such similarity. In rabbit CA III, CO₂ hydration is pH independent whereas the esterase activity displays a sigmoidal pH profile with the point of inflection at 7.5 (Koester et al, 1981). Tu et al (1986) working with bovine CA III discovered that the pH profile of k_{cat}/K_M was described by a group with pK_a 6.5, again the CO₂ hydration was pH independent. They additionally found that the apoenzyme retained esterase activity, but no CO₂ hydration

activity was evident, and thus concluded that the two activities are located at different parts of the active site. Engberg (1985) reported a very low 4-NPA activity for bovine CA III, with the apparent second order rate constant, k_{enz} , corresponding to 0.1% of the activity of bovine CA II. Interestingly, they reported no inhibition of 4-NPA hydrolysis by 1.4 mM KOCN, a powerful inhibitor of CO_2 hydration known to interact at the metal ion site. This evidence heightens speculation that the muscle enzyme has evolved away from its roots for a specific function.

1.12 BACKGROUND TO PROPOSED ACTIVE SITE STUDY

It is apparent from the preceding introduction, that the three residues at positions 64, 67 and 91 of CA III are of prime importance in determining the properties of this tissue specific isoenzyme. Certainly Arg-91 has an effect on the binding of sulphonamides and probably contributes to the resistance of this isoenzyme to sulphonamide inhibition. The presence of Arg-91 alone, however, does not account for its low CO₂ hydration activity, since the high activity tiger shark CA also has Arg-91. Positions 64 and 67 are occupied by glutamine in this ancestral CA (Bergenham, personal communication). Lys-64/Arg-64 probably exercises the most influence on catalytic activity, although site directed mutagenesis of this residue was inconclusive, and highlighted the complex nature of the catalytic mechanism in vivo, with the probable involvement of interacting ions. The third arginine at position 67 has received little attention, yet it could be the most influential. In bovine CA III, Arg-67 is directed towards the metal complex, is not bound to another residue and may be free to rotate across the active site (Eriksson, unpublished data).

Other important active site residues which may confer isoenzyme characteristic properties include Phe-198 and Cys-66. Phenylalanine at position 198, with its dense electron negative cloud probably restricts the cavity entrance, in itself though, this would not appear to be an evolutionary advantage. Of the five cysteines invariant in muscle isoenzymes, two are reactive with Ellman's reagent (Cys-183 and Cys-188). These are located on the outer portion of the molecule (Engberg and Lindskog, 1986). Cys-66 is an integral part of the active site chain 64-69, containing the aforementioned basic residues,

and there is evidence that modification of this thiol group results in enzyme activation (Engberg and Lindskog, 1986). The modified enzyme has a 3-times higher k_{cat} value and a 6-10 times higher k_{cat}/K_M value (Ren et al, 1988).

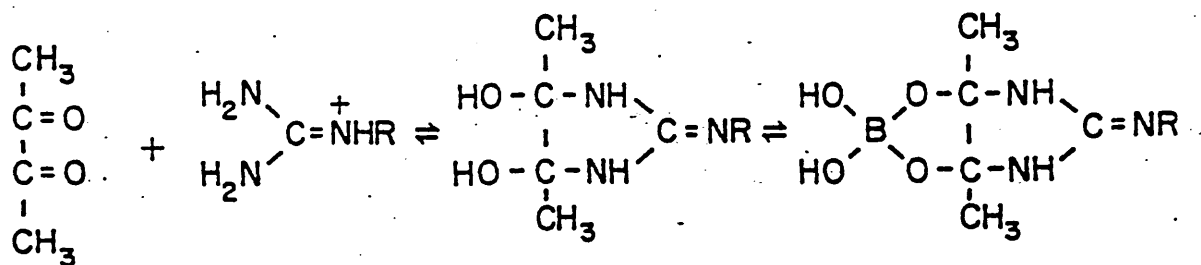
Invariant basic active site residues are often located in enzymes involved in the binding of phosphorylated substrates eg carbamoyl phosphate synthetase and creatine kinase (Borders and Riordan, 1975). Arginine has a pK_a of 12.5, so at physiological pH it has a strong electronic affinity to any negatively charged phosphate groups. The novel acid phosphatase activity of CA III, in addition to the possible separation of the hydratase and esterase sites, indicates that this isoenzyme may catalyse a unique reaction in vivo. There is some evidence that CA can be phosphorylated by the involvement of cyclic-AMP dependent protein kinase mediated by neurotransmitters like epinephrine. However, this work has not been fully verified and there are no studies specifically with CA III (Church et al, 1980; Narumi and Miyamoto, 1974).

The tissue specific location of CA III has been described. It is inconceivable that the siting of muscle CA III in type I fibres, under precise hormonal and neural control is an accident of nature. Its slow base rate change of 14 base changes/100 codons/ 10^8 years over the last 300 million years, as compared to 20 for CA II and 18 for CA I, emphasises its specialised selection throughout evolution (Tashian et al, 1980). It is worth noting that, of all the amino acid residues, arginine is one of the most tightly conserved. Assuming that the invariant basic residues are of prime importance to the physiological role of this protein, the question that arises is, "What properties do these residues confer on this protein to selectively establish it as one of the primary enzymes in this tissue?" It

remains a mystery that this isoenzyme, with an apparently low turn over number of $4 \times 10^3 \text{ s}^{-1}$ has been selected in red skeletal muscle in preference to the highly efficient CA II ($1 \times 10^6 \text{ s}^{-1}$).

In an attempt to address some of these posed problems, previous active site studies in this laboratory have centred on the effects of the arginine modifying agent, 2,3-butanedione, on the properties of the enzyme. 2,3-Butanedione (BD) is a highly selective agent for the modification of arginyl residues, provided that certain conditions of high pH and 50 mM borate buffer are employed. The borate stabilises the reversible complex of the guanidino group and butanedione as shown in Figure 1.7.

FIGURE 1.7 Arginine Modification by 2,3-Butanedione in Borate Buffer



Treatment of human and bovine CA III with 2,3-butanedione results in enhancement of both the esterase and the bicarbonate dehydration activities (Tashian et al, 1984; Chegwiddden et al, 1986). These findings are remarkable, since covalent modification of active site residues normally results in inhibition rather than activation. Sequence analysis of the modified human CA III protein revealed that active site residues 67 and 91 were modified by BD. The I_{50} for acetazolamide was reduced from 50 μM to 8 μM , thus emphasising the role of residue 91 in sulphonamide binding. The specific activities of CA I and CA II were unaffected by BD-treatment.

Modification of chicken CA III resulted in enhancement of the esterase reaction, but not the bicarbonate dehydration activity. Tryptic digestion and HPLC analysis indicated that, in this instance, Arg-91 was modified but not Arg-67. It was suggested that modification of Arg-91 may be associated with an increase in esterase activity and modification of Arg-67 with HCO_3^- dehydration activity. However, optimum assay conditions necessitated the inclusion of phosphate in the medium, an anion subsequently known to enhance activity per se.

These previous studies provided a sound basis for investigating the nature of this isoenzyme. The active site studies presented in this thesis centred on two main areas of research. The first was to extend the former BD-modifying experiments, by examining the effects of this agent without phosphate and to determine the kinetic parameters in the BD-modified enzyme. The second was to investigate the potential effects of phosphate and phosphorylated intermediates on the various activities of chicken and human CA III. It was envisaged that the work might be extended by monitoring the differences, if any, between the separate and corporate effects of BD and P_i .

Initially, it was imperative to determine the intrinsic purity of the enzyme samples extracted from red skeletal muscle, because of the precise and exacting nature of the assays. In addition, it was essential to assess various buffers for their potential effects and to select an inert medium for subsequent activity measurements. The results of these initial investigations are included for this reason and because some interesting data concerning 'CA associated' proteins emerged.

It is surprising that little attention has been focused on the possible involvement of phosphate or phosphorylated substrates, in view of the putative residues present in the active site of CA III. Christiansen and Magid (1970) demonstrated an activating effect of P_i in CA I, but not CA II; yet this phenomenon received no further attention and many subsequent studies were conducted in phosphate buffers. A similar enhancement by P_i on the HCO_3^- dehydration activity of human and chicken CA III is reported in this study and a possible mechanism is discussed. Many factors contribute to the control of P_i and phosphorylated metabolites in the active muscle cell, including the maintenance of phosphorylation potential, the glycolytic flux, blood flow and muscle type. Thus, CA III is exposed to a constantly changing pattern of P_i and phosphorylated metabolite concentration. It is postulated that the results presented in the following text are pertinent to the natural dynamic environment of this important enzyme, and may in some way assist in dispersing the cloud of mystery surrounding the function of this protein.

2.1 PURIFICATION OF CHICKEN, FROG AND HUMAN CA III FROM MUSCLE

The purification procedure employed was essentially that of Hewett-Emmett et al (1983) for isolating human muscle isoenzymes, with an additional ion-exchange chromatography step for chicken CA III described by Carter et al (1984).

Frozen chicken thighs and frog's legs were used as a source of red skeletal muscle. Approximately 10 lbs of thighs were utilised per chicken preparation and 50 pairs of frog's legs for a frog extraction. Human psoas major muscle was obtained from post mortem patients; generally two complete muscles were available, which were transported on ice and processed immediately.

The red skeletal muscle was dissected from the tendons and fat in the human tissue and from the thigh bone and tendons in the thawed chicken tissue. Any primarily white meat was discarded from the chicken legs and care was taken to remove every portion of fibre adjacent to the thigh bone, since this is particularly rich in type 1 fibres. As there is no obvious differentiation of fibre type in the frog leg muscle, all the muscle was removed from the upper and lower portions of the hind limb without further dissection.

An approximate weight of 300 g of dissected tissue was used for each enzyme purification which yielded a final harvest of about 40 mg of pure enzyme. All preparatory procedures were performed in the cold room or on ice using chilled buffers where necessary.

The weighed muscle was minced three times prior to homogenising with 2.5 volumes of 0.01 M Tris SO_4^{2+} pH 8.7 (w/v) containing 1.0 mM β -mercaptoethanol, which may be necessary to prevent

dimerisation of CA III isoenzymes (Register et al, 1978). Homogenisation, using a Waring blender in 6 x 20 s bursts, was conducted in small batches with a suitable cooling period.

The pooled crude homogenate was then agitated for at least 5 h. Globules of fat and clumped tendons which tend to aggregate during this stirring process were removed at intervals.

The cell debris was eliminated by centrifugation at 7,500 rpm for 40 min (4°C) and the supernatant collected through a funnel containing loosely packed glass wool to filter any residual fat. Following extensive dialysis against 0.005 M Tris SO_4^{2+} buffer (5 changes x 16 litres) over a 48 h period and further centrifugation, the supernatant was added to approximately 75 ml of swollen prontosil affinity resin, prepared as described in the following section.

2.1.1 Preparation of p <(2,4-diaminophenyl)azo> benzenesulphonamide (Prontosil)

The prontosil sulphonamide was prepared by diazotisation of p-aminobenzenesulphonamide and subsequent coupling to m-phenyldiamine (Yoshininobu et al, 1937). The diazotisation procedure involved adding cold sodium nitrite (4.3 g NaNO_2 in 30 ml H_2O) solution dropwise onto sulphanilamide (10 g in 17.4 ml 30% w/v HCl and 120 ml H_2O). 6.2 g of m-phenylenediamine dissolved in 10.2 ml of 10% HCl (w/v) was then added dropwise to the above cooled solution. The mixture was left on ice for several hours, filtered and the red precipitate washed with ice-cold water prior to drying in air.

2.1.2 Coupling Prontosil to the Sephadex Resin

The affinity resin was prepared by the method of Osborne and Tashian (1975) based on the technique of Hoare and Koshland (1967). Linking prontosil directly to a gel matrix results in cleavage of the sulphonamide and the distance of the coupled inhibitor from the gel matrix is not sufficient to accommodate the active site cleft of CA; so the prontosil is linked to the gel by a water soluble carbodiimide.

Fifteen grams of CM Sephadex (C50 coarse grade) were added to 450 ml of 50% acetone containing 7.5 g of prontosil. After adjusting the pH to 4.75 by the addition of 1.0 M H_2SO_4 , 7.5 g of 1-(3-dimethylaminopropyl)3-ethylcarbodiimide HCl (freshly dissolved in 15 ml of H_2O) was added dropwise with continuous gentle stirring. The pH was maintained at 4.75 throughout this latter process. The reaction mixture was gently stirred overnight at room temperature.

The gel was washed with 4 litres of 100% acetone, followed by 4 litres of 50% acetone and finally by hot water adjusted to pH 10 with sodium carbonate. The gel was regenerated between preparations by batch washing with one litre of hot 1 M sodium carbonate, followed by hot water, cold distilled water and finally buffer. With careful handling the resin can last several years with complete effectiveness.

The efficiency of each batch of affinity resin was estimated with commercial bovine CA II, by coupling a known quantity of enzyme to the resin. The amount of unbound enzyme was subsequently compared to the amount of enzyme eluted with azide, by measuring absorbance at 280 nm. A percentage binding capacity of 95% was not uncommon.

2.1.3 Elution of CA III from Sulphonamide Affinity Resin

The affinity resin added to supernatant collected after homogenisation of muscle binds to cytosolic muscle CA III, CA I and CA II from disrupted erythrocytes, possibly some membrane CA IV and also non-specific protein. The elution of specific isoenzymes is mediated by the differential binding to sulphonamides (Chegwidden and Shelton, 1987; Chegwidden, in press). Thus, CA III with the greatest resistance to sulphonamides is readily eluted with mild salt treatment, whereas CA II elution requires more extreme treatment. The problems encountered with non-specific binding of muscle proteins are discussed at length in section 3.

The Sephadex with bound chicken, frog or human CA III was washed with approximately six litres of chilled 0.05 M Tris SO_4^{2+} buffer pH 8.7 to remove non-specifically bound protein until the $A_{280\text{nm}}$ <0.02. It was then poured into a 1.5 cm x 75 cm glass column. A linear gradient of 0.05 M Tris SO_4^{2+} /0.2 M Na_2SO_4 was applied

to the chicken CA and frog "CA" bound gels, and a 0.05 M Tris SO_4^{2+} /0.2 M KI gradient was applied to the gel with bound human CA III at a rate of 30 ml h^{-1} . Aliquots of 4 ml were collected and tested for carbon dioxide hydration activity using the Bromothymol Blue (BTB) spot test (2.1.3).

BTB-positive chicken fractions were pooled and vacuum dialysed as described by Watts and Moreland (1970) against 0.05 M Tris Cl^{-1} pH 8.7 (Chegwidden et al, 1984). Reduction of volume from \approx 70 ml to 2 ml generally took 24 h.

The pooled BTB-positive human fractions were dialysed against 5 litres of buffer immediately after collection, since prolonged complexing with KI results in impaired catalytic activity. The dialysate was then vacuum dialysed against 0.05 M Tris SO_4^{2+} to approximately 2 ml.

The frog BTB-'positive' samples were vacuum dialysed against 0.05 M Tris SO_4^{2+} pH 8.7.

2.1.4 Bromothymol Blue (BTB) Spot Test

The Bromothymol Blue test, developed in Tashian's laboratory (Tashian, 1969) from the original method of Pihar (1965), is a reliable method for determining positive fractions when specifically isolating carbonic anhydrase; although care must be taken that fractions do not become acidic. It allows the rapid detection of CA in a hundred or more samples at a time.

Five μl of each fraction were loaded as discrete spots onto Whatman Grade 1 Chr filter paper soaked in 0.15% BTB dissolved in 0.06 M barbitol buffer pH 8.7. The paper was subsequently placed in a trough on ice and covered with a perspex lid fitted with an inlet.

After applying the aliquots, the trough was gassed with 100% CO₂ for 20 s and the inlet sealed. A bright yellow spot after 5 s indicated the formation of carbonic acid and hence the presence of CA.

2.1.5 Ion-exchange Chromatography of Chicken CA III

The vacuum dialysed chicken sample after centrifuging at 10,000 rpm for 15 min, was passed through a 0.5 x 10.0 cm DEAE-Tris acryl M anion exchange column equilibrated with 0.05 M Tris Cl⁻ pH 8.7, at a rate of 10 ml h⁻¹. Chicken CA III, with a basic pI_E (Holmes, 1976) is readily separated from any contaminating myoglobin by this procedure. The BTB active fraction, usually contained in one 4 ml sample, was vacuum dialysed against 0.05 M Tris SO₄²⁺ to 1 ml. This purification step was omitted for the frog and human preparations. The column was regenerated periodically by the application of 1 M aqueous sodium chloride followed by treatment with an appropriate protease.

2.1.6 Separation by Gel Filtration

The final preparatory step involved fractionation by gel permeation using a 'purpose built' 100 x 1.5 cm glass column packed with G75 Sephadex. The manufacturer's product information suggest that the exclusion limits for G75 are 3,000 and 80,000. Calibration of the column using markers of known molecular mass indicated that the working margins were very much less. Nonetheless, carbonic anhydrase with a RMM of 30,000 was adequately separated from most contaminating proteins, provided that the initial sample was fairly pure and that two fractions each side of the BTB positive peak from the affinity

resin were discarded. The whole question of sample purity and CA-associated proteins is discussed in section 3.

The reduced volume samples, preferably 1 ml or less, were loaded carefully onto the surface of the equilibrated gel together with a blue dextran marker and eluted with 0.05 M Tris SO_4^{2+} buffer at a rate of 40 ml h^{-1} . Fractions of 4 ml were collected after the dye had passed through the column. The BTB-positive fractions were pooled and vacuum dialysed as before.

All isoenzymes were stored at 4°C in buffer at a concentration of over 10 mg ml^{-1} . Samples stored for prolonged periods were filtered through a sterile millipore filter to avoid bacterial contamination.

Enzymes were tested for purity by SDS-PAGE and the concentration was determined by measuring the absorbance at 280 nm.

2.2 PURIFICATION OF HUMAN CA I AND CA II FROM WHOLE BLOOD

Erythrocytes were extracted from whole blood by centrifugation at 3,000 rpm for 20 min. After two washes with 0.9% NaCl and respinning, the red blood cells were lysed by the addition of 2 volumes of water (Osborne and Tashian, 1975). The pH was adjusted to 6.0 with 0.5 M H_2SO_4 and the cell debris removed by centrifugation at 15,000 rpm for 15 min. The lysate was extensively dialysed against buffered water pH 9.0 and 0.2 M $\text{Na}_2\text{SO}_4^{2+}$ pH 9.0 before addition to the affinity resin.

2.2.1 Elution of CA I from the Prontosil-linked Affinity Resin

The resin was poured into a 100 x 2 cm column and non-specifically bound protein eluted by washing with 0.2 M $\text{Na}_2\text{SO}_4^{2+}$ in 0.1 M Tris SO_4^{2+} pH 9.0 until the absorbance at 280 nm was zero. The CA I isoenzyme was uncoupled from the resin by applying 100 ml 0.4 M KI in 0.1 M Tris SO_4^{2+} pH 7.0 buffer, followed by 100 ml of the same buffer at a rate of 40 ml h^{-1} . The CA II remains bound to the resin by this treatment. Four millilitre fractions were assayed for CA activity and the active fractions pooled, dialysed and then vacuum dialysed. High levels of homogeneity were obtained without the need for further purification.

2.2.2 Isolation of CA II

Carbonic anhydrase II has a strong affinity for sulphonamides and requires extreme treatment to remove it from the resin. Thus, after CA I elution, CA II recovery was achieved by the application of

100 ml of 0.4 M NaN_3 in 0.75 M Tris SO_4^{2+} , pH 7.0 followed by 100 ml of the same buffer. This is an exceedingly hazardous procedure requiring adequate fume cupboard facilities and every precaution was taken to ensure that the azide never became acidic. BTB-positive fractions were pooled and promptly dialysed against 0.005 M Tris SO_4^{2+} pH 8.7 and vacuum dialysed to 1-2 ml for subsequent storage.

2.3 EQUINE CA I, II AND III

Horse CA I and CA II were kindly donated by Dr H F Deutsch for kinetic and inhibition studies. Equine CA III was isolated by the same method as that for human CA III.

New carbonic anhydrase preparations were always assessed for purity despite the fact that the isolation protocol was identical for each batch. An apparatus for discontinuous vertical flat-sheet polyacrylamide gel electrophoresis was made according to the specifications outlined by Reid and Bielecki (1968). Using the buffer system developed by Laemmli and Favre (1973) for separating phage T4 proteins by disc electrophoresis, reproducible and accurate results were obtained with pg quantities of protein. Moreover, the flat sheet method allowed precise comparisons of bands across the gel. A photograph of the apparatus is shown below.

2.4.1 SDS-PAGE Buffers

Acrylamide Stock Solution

30.0 g acrylamide (care)

0.8 g bisacrylamide

Made up to 100 ml with distilled water

Separating Gel Buffer

0.4 g sodium dodecyl sulphate

Made up to 100 ml with 1.5 M Tris-HCl

(18.2 g Trizma base adjusted to pH 8.8 with 0.5 M HCl)

Stacking Gel Buffer

0.4 g sodium dodecyl sulphate

Made up to 100 ml with 0.5 M Tris-HCl

(6.1 g Trizma base adjusted to pH 6.8 with 1 M HCl)

Tank Buffer

10.0 g sodium dodecyl sulphate

144.0 g glycine (1.92 M)

30.3 g Trizma base (0.25 M) pH 8.3

Made up to 1 litre with distilled water and diluted 1:10

prior to use

Sample Buffer

10.0 ml glycerol (20%)

2.0 g sodium dodecyl sulphate (4%)

0.01 g bromophenol blue

5.0 ml 2-mercaptoethanol (10%)

Made up to 50 ml with 0.25 M Tris-HCl (1.5 g Trizma base
adjusted to pH 6.8 with 1 M HCl)

All the stock solutions were kept for several months at 4°C,
apart from the sample buffer which was frozen in small aliquots.

2.4.2 Preparation of the Gel

The two glass plates visible in Plate 2.1 were sandwiched
together and sealed with molten 1.5% agar, prior to introducing the
12.5% separating gel. This running gel was prepared by mixing the
following solutions in the order listed:

8.1 g acrylamide stock solution

1.9 ml distilled water

5.0 ml separating gel buffer

5.0 ml freshly prepared ammonium persulphate (1%)

25 μ l N,N,N',N'-tetramethylethylenediamine (TEMED)

The separating gel was overlaid with isopropanol until set
(\approx 30 min). After polymerisation, the alcohol was decanted, the gel
surface washed with water and stacking gel added, up to the top of the

notched glass plate. A comb containing 12 well moulds was pushed down into the gel surface.

The stacking gel with a 5% acrylamide content was prepared as follows:

3.2 ml acrylamide stock solution

6.8 ml distilled water

5.0 ml stacking gel buffer

5.0 ml 1% ammonium persulphate

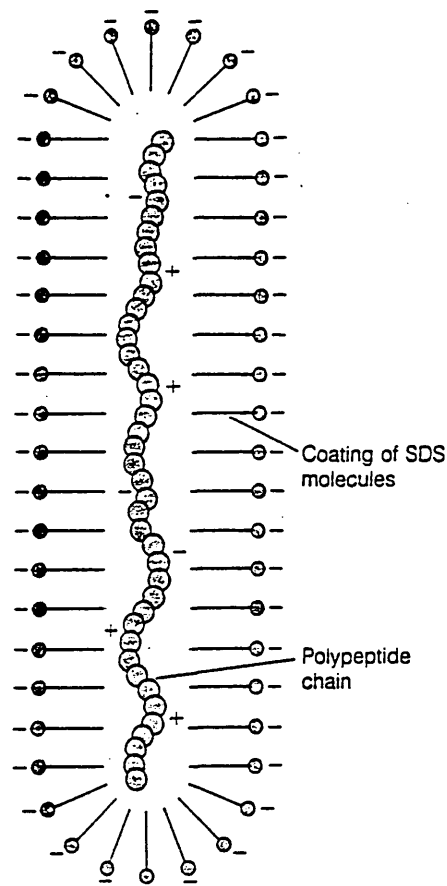
25 μ l TEMED

When polymerisation of the stacking gel was complete, the spacers and comb were removed and the plate clamped to the apparatus as shown in the photograph. The upper and lower chambers were filled with approximately 800 ml of diluted tank buffer prior to sample loading.

2.4.3 Electrophoresis of Protein Samples

SDS-PAGE depends on the uniform augmentation of negative charge along each polypeptide (Figure 2.1). This necessitates total denaturation of the native protein in the presence of high concentrations of SDS, which was achieved by boiling each sample for two minutes with an equal volume of sample buffer.

FIGURE 2.1 Modification of Protein by Sodium Dodecyl Sulphate



Hamilton syringes were used for sample application and generally the sample volumes were of the order of 5-15 μ l corresponding to 0.5-7.5 μ g of protein. The presence of glycerol prevents dissolution of the test samples and the dye aids accurate loading. The electrophoreses were run at a constant voltage of 150 v with normal polarity for about 3 h or until the dye front almost reached the bottom of the gel.

2.4.4 Fixing, Staining and Destaining

Gels were fixed in destaining buffer (methanol : H₂O : glacial acetic acid; 1 : 1 : 0.2) for one hour then stained in 0.5% Coomassie Blue dissolved in the destaining buffer for 1-2 h. After aspiration of the stain the gels were washed with water and allowed to destain in the above buffer for up to a week. Permanent photographic records were kept when appropriate.

2.5 ANODIC DISCONTINUOUS POLYACRYLAMIDE GEL ELECTROPHORESIS (DISC-PAGE)

The system adopted for anodic DISC-PAGE was essentially that described for SDS-PAGE (2.4), except that no SDS was included in the separating, stacking or tank buffers. Proteins were analysed in their native state in 0.25 M Tris HCl buffer pH 6.8 with 20% glycerol and 0.02% Bromophenol Blue. No heat treatment was necessary. The separating and stacking gels were prepared as for SDS-PAGE with 1.5 M Tris HCl pH 8.8 replacing the separating gel buffer and 0.5 M Tris HCl pH 6.8 replacing the stacking gel buffer.

2.6 CATHODIC DISC-PAGE

(Polyacrylamide Gel Electrophoresis - Pharmacia Fine Chemicals)

The cathodic DISC-PAGE System was as described above for anodic DISC-PAGE, except for the gel buffers.

Stacking gel buffer: 0.06 M KOH in 0.062 M acetic acid,
pH 6.8

Separating gel buffer: 0.06 M KOH in 0.375 M acetic acid,
pH 4.3

Electrophoresis buffer: 0.35 M alanine in 0.14 M acetic
acid, pH 4.5

2.7 HORIZONTAL GEL ELECTROPHORESIS

In spite of the extensive literature covering many different aspects of carbonic anhydrase research, no reliable method of separating native carbonic anhydrase isoenzymes by flat-bed electrophoresis has been reported. A protocol was developed in this laboratory to discretely separate the cathodally migrating CA III isoenzymes from these isoenzymes bound to inhibitors or modifiers or from other anodally migrating forms.

One percent type II agarose with medium EEO (0.16-0.19) dissolved in 0.02 M Tris HCl pH 4.5/30 mM glycine (Holmes, 1976) was found to be most effective. The gels were cast on 10 x 10 cm glass plates and electrophoresed at 150 v for 4-5 h in the above buffer. Plates were stained by immersion in 0.5% Coomassie Blue dissolved in destaining buffer (2.4.4) for 20 min followed by destaining overnight. The agarose was dried onto the plates by placing them on foil over a water bath set at 60°C. This critical temperature allowed warming without disruption or distortion of the gel system.

2.7.1 Modification by Dansylamide

Chen and Kernohan (1967) showed that the fluorescent sulphonamide 5-dimethylaminonaphthalene-1-sulphonamide (DNSA-dansylamide) forms a highly fluorescent 1:1 complex with CA II from bovine erythrocytes. After treatment with dansylamide, CA samples and frog protein samples were analysed by electrophoresis as described in 2.7. The resulting spots were visualised by examination under a UV lamp and by staining.

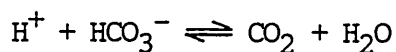
DNSA solution (50 μ M) was prepared by dissolving 0.5 mg in 2 ml of 0.1 M NaOH. This alkaline solution was subsequently diluted 1:20 in 10 mM Tris SO_4^{2+} pH 8.9 prior to use (Drescher, 1978). CA and frog samples adjusted to about 50 μ M were mixed with equal volumes of DNSA at room temperature and applied to the agarose gels prior to electrophoresis.

2.8 ENZYME ACTIVITY MEASUREMENTS

2.8.1 Bicarbonate Dehydration Activity

The rate of CO_2 formation from NaHCO_3 (dehydration reaction) was followed at 2°C by a pH-stat technique (Hansen *et al*, 1966; Magid, 1968). The pH-stat assay system consisted of a Radiometer autoburette linked to an automatic titrator and recorder, and a methanol cooling system. The complete assembly is shown in the photograph (Plate 2.2). The KCl calomel reference electrode and glass electrode were housed in a central reaction vessel fitted with a stirrer along with a titrant inlet. This entire vessel was surrounded by a cooling jacket connected to a methanol bath, and the base was fitted with a sintered filter connected to a nitrogen supply.

Carbonic anhydrase bicarbonate dehydration follows the reaction:



CA

As the reaction proceeds protons are absorbed and the medium becomes gradually basic. The pH-stat automatically replaces any lost protons by titrating acid to maintain a constant pH; thus the volume of acid titrated is directly proportional to the progress curve of the reaction.

The autoburette was set to a fixed pH end point of 7.1, except when the effect of pH was being examined and the titrant maintained at 0.1 M H_2SO_4 apart from kinetic studies when concentrations of 0.5 M, 1.0 M and 2.0 M H_2SO_4 were necessary. The nitrogen pressure was set to maintain an even flow through the sinter and thus remove CO_2 formed in the reaction. It was always set at a limiting level and remained unaltered during the day's measurements. The complete removal of CO_2 during the course of the assay eliminates the need to correct the experimental data for the back reaction.

To minimise the uncatalysed 'blank' reaction, strict attention was paid to keeping all reagents cooled on ice prior to use and the methanol bath, set at 2°C , was checked frequently during assay measurements. Blank traces were recorded at regular intervals.

An example of a typical reaction mixture is shown below, any alterations to the standard assay are described in the text.

| | |
|---|--------|
| Distilled water/other additions | 7.6 ml |
| 0.05 M hepes buffer pH 7.1 | 2.0 ml |
| 1 M NaHCO ₃ (freshly prepared) | 0.3 ml |
| Enzyme (0.02-0.1 mg)/H ₂ O blank | 0.1 ml |

TOTAL 10.0 ml

Reproducible results were achieved by accurate pipetting, using blow-out pipettes for large volumes and Hamilton syringes for volumes below 0.5 ml. Assays were performed by initially pipetting buffer, water and/or other additions into the vessel, followed by the substrate. The addition of substrate results in a substantial alteration of pH, so at this point the burette was operated without the recorder to bring the pH back to 7.1, and thus allow a smooth 'take off' upon the final inclusion of enzyme. The recorder was connected immediately following the enzyme addition and the acid titrated was usually monitored for approximately 5 min. In inhibition studies, necessitating preincubation of enzyme and inhibitor, the reaction was initiated by the addition of substrate which incurred a slight delay to allow equilibration of bicarbonate before recording could commence.

a) Calculation of Enzyme Activity

Figure 2.2 shows a typical pH-stat assay trace. Assays 1 and 2 were blank traces and 3 a test trace with CA III in the presence of 25 mM phosphate.

Determination of acid titrated

Chart speed = 10 s cm⁻¹

Pen movement x 2.5 = 1 ml acid for full scale deflection

Vol acid titrated per unit time = Vol over 6 cm ml min⁻¹

Most measurements were taken after 0.1 ml acid had been titrated and a tangent was drawn to the trace at this point.

Enzyme Activity

Titrant = 0.1 M H₂SO₄

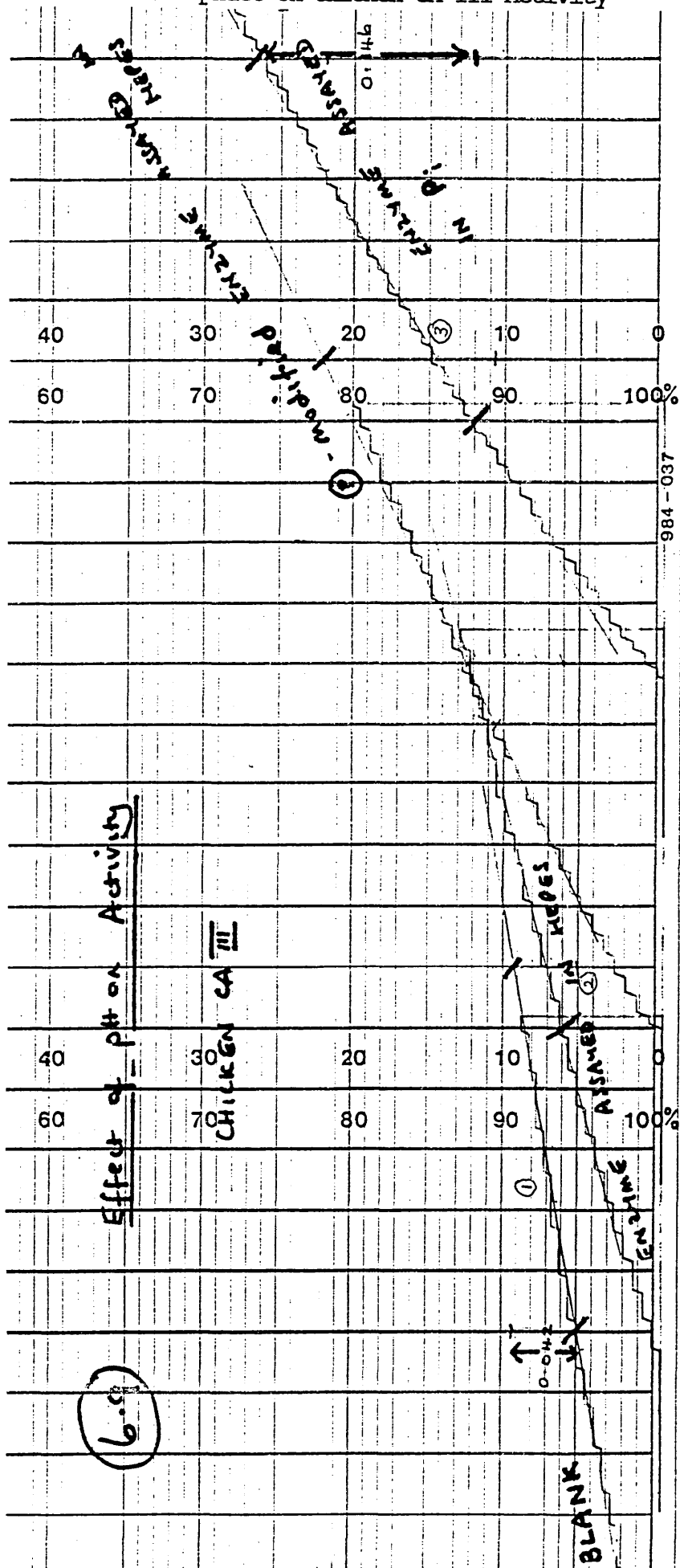
1 ml 0.1 M H₂SO₄ contains 0.1 mmol

There are 2H⁺ equivalents from H₂SO₄ per mol of product formed.

Therefore SPECIFIC ENZYME ACTIVITY =

$$\frac{(\text{ml min}^{-1} \text{ test} - \text{ml min}^{-1} \text{ blank}) \times 10^{-2}}{2 \times \text{mg enzyme}} \mu\text{mol product min}^{-1} (\text{mg enzyme})^{-1}$$

FIGURE 2.2 Typical pH-stat Assay Trace Showing the Effect of Phosphate on Chicken CA III Activity



b) Sample Size and Standard Deviation

Initially each assay was repeated at least five times but the requirement to conduct comparative studies during the same period necessitated the reduction of sample number to two. The prohibitive cost of some phosphorylated compounds allowed only single assays. However, all the investigations were repeated at least three times and some of the kinetic studies as many as twenty times. A degree of manual dexterity can be achieved with practice. Table 2.1 shows the scatter from a typical set of results.

TABLE 2.1 Statistical Analysis of the Bicarbonate Dehydration Reaction

| pH | n | Treatment | Activity | Mean | SD | +/- 2SE* | % Mean |
|------|---|-----------|--------------------------------------|-------|-------|----------|--------|
| 7.1 | 5 | phosphate | 7.71 6.80 7.23 6.80 7.00 | 7.108 | 0.380 | 0.340 | 4.78 |
| 7.1 | 5 | Hepes | 4.14 3.74 3.17 3.17 2.94 | 3.432 | 0.494 | 0.442 | 12.88 |
| 7.6 | 5 | phosphate | 2.29 2.57 2.34 2.23 2.43 | 2.372 | 0.133 | 0.118 | 4.98 |
| 7.6 | 5 | Hepes | 1.29 1.14 1.29 1.23 1.26 | 1.242 | 0.062 | 0.056 | 4.50 |
| 7.35 | 3 | phosphate | 4.34 4.09 4.26 | 4.230 | 0.128 | 0.148 | 3.50 |
| 7.35 | 3 | Hepes | 1.80 1.89 1.86 | 1.850 | 0.046 | 0.054 | 2.92 |

* +/- = 95% confidence limits

2.8.2 Esterase Activities

1 Hydrolysis of 4-nitrophenyl Acetate (Armstrong et al, 1966)

Esterase activity towards 4-nitrophenyl acetate (4-NPA) was estimated by a modification of the method of Verpoorte et al (1967). The standard assay is as follows:

Test Cuvette

| | |
|--------------------------------------|--------|
| 0.12 M sodium diethylmalonate pH 7.2 | 0.2 ml |
| 0.005 M 4-NPA (freshly prepared) | 0.2 ml |
| Distilled water/other additions | 0.3 ml |
| Enzyme (0.3-0.6 mg CA III) | 0.1 ml |
| TOTAL | 0.8 ml |

The reaction was initiated by the addition of enzyme to the test cuvette, and the increase in absorbance was followed at the wavelength of the isobestic point of the nitrophenol and conjugate nitrophenolate ion (348 nm) for approximately 20 min. Steady state measurements were monitored in a dual beam Cary 210 equipped with two thermostated cell holders adjusted to 25°C.

The low solubility of 4-NPA in water necessitated dissolving the ester in 1 ml of acetone initially, with a subsequent slow addition of water. However, even with this prerequisite, 5 mM was the maximum substrate concentration attained. Thus, for kinetic measurements by replacing 0.3 ml of water for 4-NPA, a maximum of 3.125 mM was attainable.

a) Calculation of Enzyme Activity

Progress curves for the esterase reaction of CA III did not deviate appreciably from first order kinetics, in contrast to the findings of Verpoorte (1967) for CA I and CA II, who suggested that product inhibition might be a contributory factor. Nonetheless, tangents were drawn to calculate initial velocities and the rates appropriately adjusted for chart speed and range.

Molar Absorbance Coefficients

$$\text{4-nitrophenol and 4-nitrophenolate ions} = 5.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$$

$$\text{4-NPA} = 0.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$$

SPECIFIC ENZYME ACTIVITY =

$$\frac{A_{348\text{nm}} \text{ min}^{-1}}{5.0 \times \text{mg enzyme} \times 10^3 \times 0.8} \quad \text{mmol min}^{-1} (\text{mg enzyme})^{-1}$$

b) Sample Size and Standard Deviation

Comparisons of kinetic data for different treatments required precise measurements on the same day. Each assay took at least 25 min and utilised up to 0.6 mg of enzyme; consequently it became expedient to reduce sample size to a minimum. In order to estimate the error of the assay, the same sample was assayed ten times under the same conditions. The results are listed in Table 2.2.

TABLE 2.2 Statistical Analysis of a Typical Set of Esterase
(4-NPA Hydrolysis) Results

| Sample No | Activity nmol min ⁻¹ mg ⁻¹ | Mean | SD | SE |
|-----------|---|-------|-------|-------|
| 1 | 0.58 | 0.627 | 0.036 | 0.014 |
| 2 | 0.64 | | | |
| 3 | 0.64 | | | |
| 4 | 0.63 | | | |
| 5 | 0.57 | | | |
| 6 | 0.63 | | | |
| 7 | 0.63 | | | |
| 8 | 0.69 | | | |
| 9 | 0.61 | | | |
| 10 | 0.66 | | | |

Standard Error of the Mean = 0.014

ie +/- 0.028 is within 95% confidence limits,
this is 4.5% of the mean.

2 Hydrolysis of 2-Nitrophenyl Acetate (Verpoorte et al, 1967)

Hydrolysis of 2-NPA was assayed essentially as described for 4-NPA, except that absorbance change was followed at the wavelength of maximum difference between the nitrophenol and phenolate ion.

Molar Absorbance Coefficients

$$\text{2-nitrophenol} = 2.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$$

$$\text{2-NPA} = 0.14 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$$

SPECIFIC ENZYME ACTIVITY =

$$\frac{A_{372} \text{ min}^{-1}}{2.26 \times \text{mg enzyme} \times 10^3 \times 0.8} \quad \text{mmol min}^{-1} \text{ mg}^{-1} \text{ enzyme}$$

3 Hydrolysis of β -naphthyl Acetate (Tashian, 1969)

0.1-1.0 ml 5 mM β -naphthyl acetate

(dissolved in 1 ml acetone made up
to 25 ml with distilled water)

1.0 ml 0.1 M Tris-HCl pH 8.5 containing 4% p-dioxane
and 1% Brij 35

0.1 ml Enzyme (^ 0.1 mg)

up to 0.9 ml Distilled water

TOTAL 2.1 ml

After incubating at 37°C for 30 min the reaction was stopped by adding 1.0 ml 4 mM acetazolamide, then 1.0 ml freshly prepared 0.2% diazotised p-chloro-o-toluidine (Fast Red TRN) was added and allowed to develop for 45 min.

0.5 ml of sample was subsequently diluted with 1.5 ml 95% ethanol and the absorbance measured at 490 nm against a similarly treated reagent blank.

4 Hydrolysis of HNTAS

a) Preparation of 2-hydroxy-5-nitro- α -toluenesulphonic Acid

Sultone (HNTAS) (Zaborsky and Kaiser, 1966; Kaiser and Lo, 1969)

A mixture of 41.3 g sodium bisulphite and 49.2 g 2-hydroxy benzyl alcohol was dissolved in \approx 500 ml water, and refluxed for 6 h. Excess water was removed by distillation and then the solution was evaporated to dryness using a rotary evaporator.

The white powdered, 2-hydroxy- α -toluene sulphonate was extracted with ethanol. To obtain HNTAS, 40 g of white residue was added to 320 g phosphorus oxychloride. The mixture was carefully heated for approximately 1 h (\approx 125°C) and the distillate removed. The creamy coloured residue was carefully transferred into \approx 600 ml ice water, and left in contact with the water for 4 h. After suction filtering, the HNTAS was air dried and recrystallised from ethanol.

b) Assay Procedure (Sanyal et al, 1983)

| | |
|---------------|---|
| 0.05-0.50 ml | 5 mM HNTAS (0.011 g dissolved 1 ml acetone and made up to 10 ml water) |
| 0.10 ml | 0.1 M barbitone buffer pH 7.4 |
| 0.01 ml | Enzyme (0.05 mg) |
| Up to 0.55 ml | Distilled water |

TOTAL 0.80 ml

The formation of the hydrolysis product, a substituted 4-nitrophenol, was measured at 410 nm. The reaction is rapid and was generally monitored for two minutes at 25°C, against a reagent blank using a Cecil 5000 series dual beam spectrophotometer (CE 5501).

c) Calculation of Enzyme Activity

The molar absorbance coefficient was difficult to determine, so enzyme activity was expressed as absorbance change at 410 nm per second per mg enzyme.

2.8.3 Acid Phosphatase Activity

The 4-nitrophenyl phosphatase activities of CA III and associated proteins were estimated in accordance with the method described by Koester et al (1981).

Reaction Mixture

| | |
|--|--------|
| 0.05 M 4-nitrophenyl phosphate in 0.25 M | |
| Sodium Succinate pH 5.3 | 0.1 ml |
| 0.2 M Sodium Borate pH 5.3 | 0.1 ml |
| 0.25 M Sodium Succinate pH 5.3 | 0.3 ml |
| Enzyme (up to 6mg)/Distilled water for blank | 0.1 ml |
| Distilled water | 0.4 ml |
| TOTAL | 1.0 ml |

The above reaction mixture was incubated at 30°C. 0.2 ml was removed at time 0, then at 30 min, 60 min and 120 min thereafter, and added to 0.8 ml of 2 M NaOH. The absorbance of any 4-nitrophenoxide ion, released by phosphatase activity was estimated at 405 nm against a water blank.

a) Calculation of Enzyme Activity

Figure 2.3 illustrates the acid phosphatase activity of one particular frog sample over three hours. The absorbance values plotted have been corrected for the blank reactions.

$$\text{4-nitrophenoxide ion} = 1.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$$

(Pullan and Noltman, 1985)

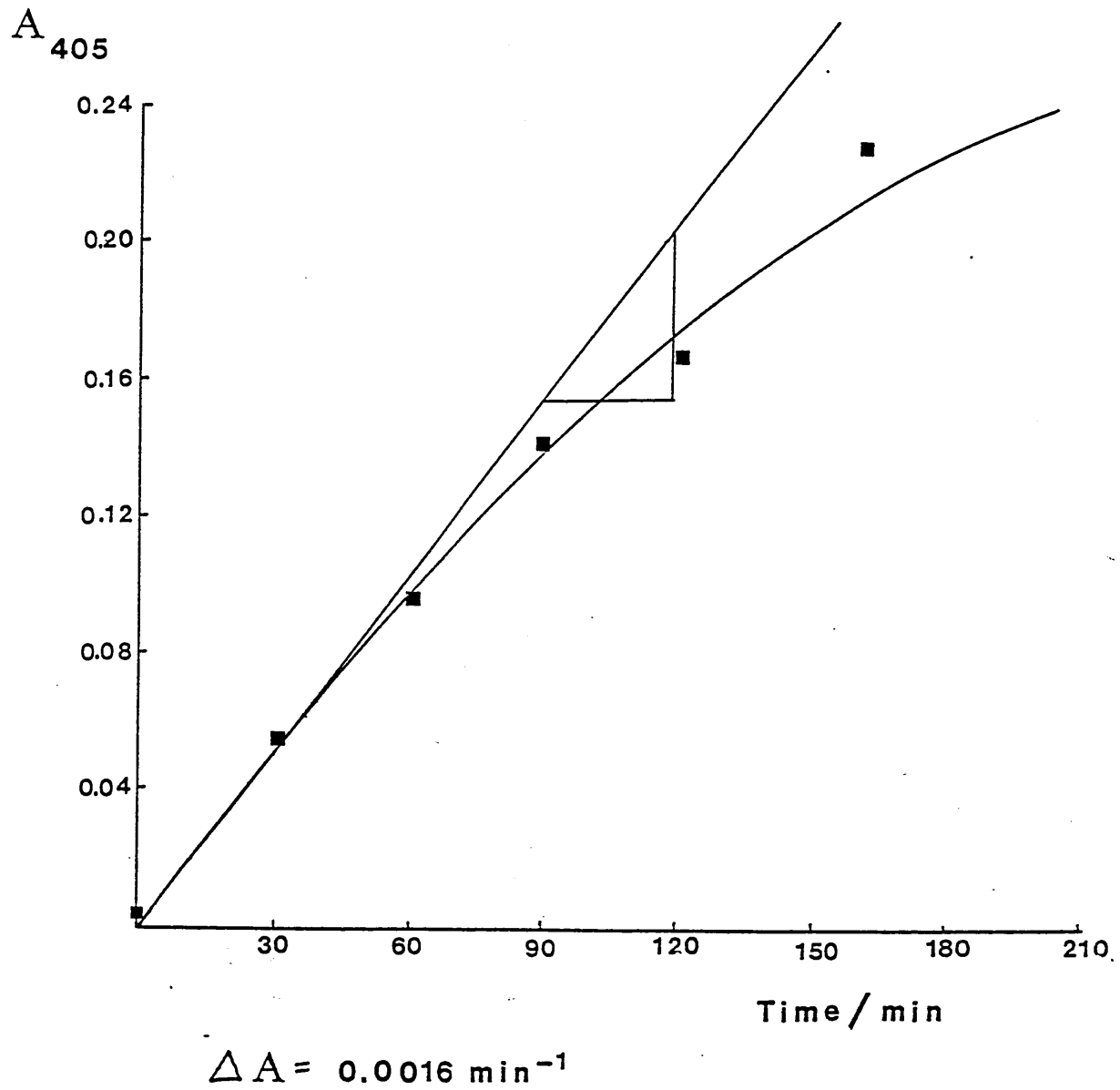
SPECIFIC ENZYME ACTIVITY =

$$\frac{A_{405\text{nm}} \text{ min}^{-1}}{18 \times \text{mg enzyme} \times 10^3} \quad \text{nmol min}^{-1} \text{ mg}^{-1}$$

$$\begin{aligned} \text{Activity of the frog protein} \\ \text{from Figure 2.3} &= \frac{0.0016 \text{ nmol min}^{-1}}{18 \times 10^3} \\ &= 0.089 \text{ nmol min}^{-1} \end{aligned}$$

$$\text{SPECIFIC ENZYME ACTIVITY} = 0.071 \text{ nmol min}^{-1} \text{ mg}^{-1}(\text{protein})$$

FIGURE 2.3 Graph to Show Acid Phosphatase Activity of Frog Protein Sample



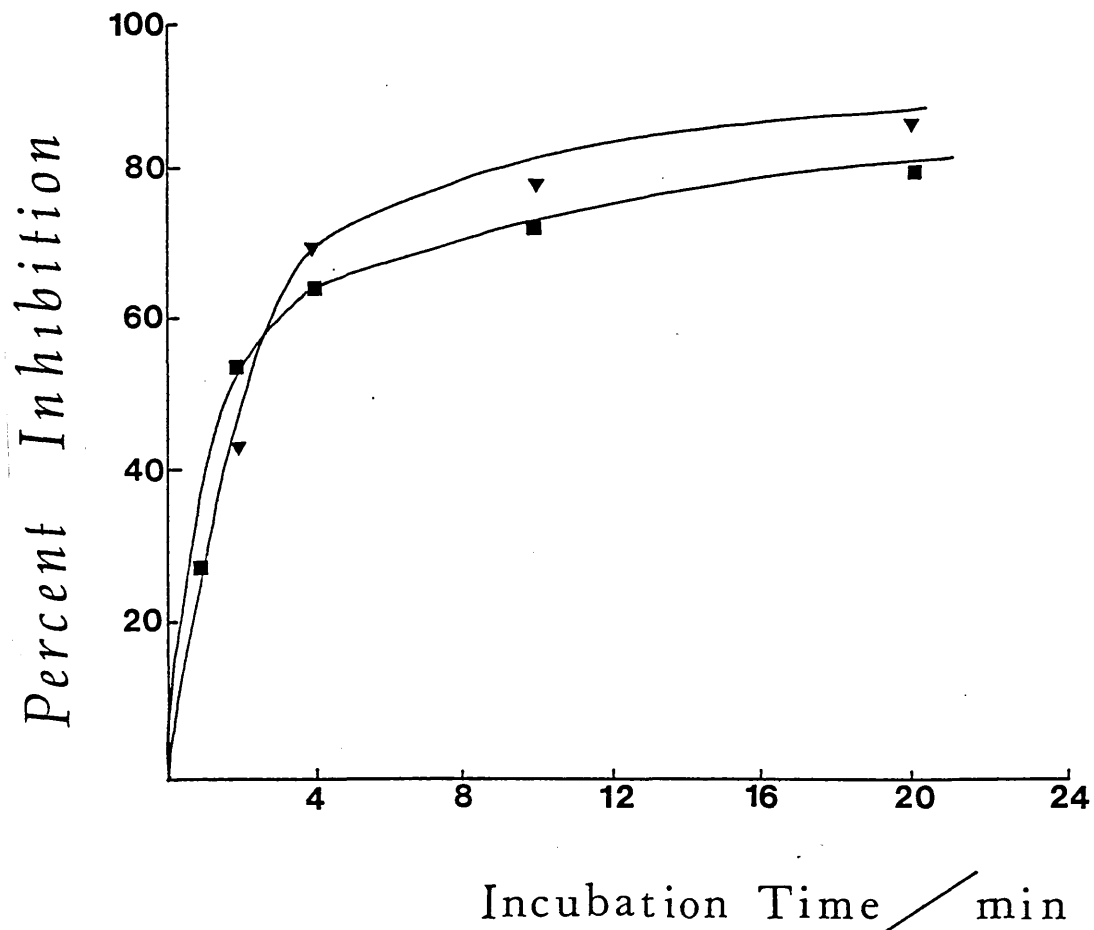
2.9 INHIBITION AND ACTIVATION STUDIES OF ENZYME ACTIVITY

A number of buffers, anions, phosphorylated metabolites and inhibitors were incorporated into the pH-stat system and esterase assays; and their effects monitored. Initially these reagents were included directly into the assays, with the exception of the sulphonamides, but latterly, the effects of preincubation of these metabolites with the enzyme were examined. The percentages of activation and inhibition were calculated with reference to suitable controls. Thus, the percentage of phosphate activation would be calculated with reference to activity measurements in hepes only.

Inhibition by sulphonamides results in a slow conformational change, so preincubation of inhibitor and enzyme is essential and the time of the incubation period critical. Figure 2.4 illustrates the time course of inhibition of human CA III with ethoxzolamide and acetazolamide. An arbitrary time of three minutes was chosen for all sulphonamide inhibition studies and this preincubation time was precisely monitored using a stop watch.

Details of the chemicals investigated are given in the materials and suppliers section (2.17). The majority were dissolved in doubly distilled water. The sulphonamides, however, exhibit low aqueous solubilities particularly at neutral pH, and consequently required varying treatments (Maren, 1984). Ethoxzolamide was initially dissolved in 1 ml DMSO, pH adjusted then diluted appropriately, whereas acetazolamide was solubilised in a small volume of water pH 9.0, then brought back to neutrality with dilute acid.

FIGURE 2.4 Graph to Show the Time Course of Inhibition of Human CA III with Ethoxolamide and Acetazolamide



Bicarbonate Dehydration Activity was measured at pH 7.1, 2°C after varying periods of incubation with 0.1 mM ethoxolamide (▼—▼) and 0.1 mM acetazolamide (■—■).
0.6 μ M human CA III

2.10 2,3-BUTANEDIONE (DIACETYL) MODIFICATION

The conditions employed for 2,3-butanedione (BD) modification are known to selectively modify arginine residues (Borders and Riordan, 1975; Chegvidden et al, 1986). These conditions described by Chegvidden et al (1988) for chicken CA III, entail reacting enzyme (40 μ M) with 25 mM 2,3-butanedione in 50 mM borate buffer pH 8.3 at 25°C, in the dark. The formation of the borate-BD-enzyme complex is biphasic, with an initial binding of BD, followed by a slower conformational change over a twenty four hour period. To maximise arginine modification within the working day, CA was reacted with freshly prepared 50-70 mM 2,3-butanedione in 50 mM borate buffer pH 8.3 for a minimum of 3 h at room temperature.

In view of the long incubation period at room temperature, a protocol was adopted to ensure that adequate controls were always included. The method is outlined in Table 2.3. The BD was generally diluted 1:1 to allow the flexibility of including other reagents; for instance BD and phosphate could both be included in a competitive situation.

Experimental Protocol for Determination of the Effects of

BD-modification and phosphate

| | 125 mM BD in 100 mM borate pH 8.3 | 5 mM Tris pH 8.3 | Enzyme | 250 mM phosphate |
|---------------------------|---|---------------------|---------|---------------------|
| BD*-modified enzyme | 0.55 ml | 0.45 ml | 0.1 ml | -- |
| BD*-control | 0.55 ml | 0.55 ml | -- | -- |
| Enzyme control | -- | 1.00 ml | 0.10 ml | -- |
| Phosphate-modified enzyme | -- | 0.45 ml | 0.10 ml | 0.55 ml |

Preparation of BD Solution* - 125 μ l BD was added to 10 ml
100 mM borate buffer pH 8.3, readjusted to pH 8.3 with 6 M NaOH, then
made up to 11.4 ml with buffer. Final concentration BD:125 mM.

2.11 MODIFICATION WITH PHENYL GLYOXAL

Pullan and Noltman (1985) reported inhibition of the phosphatase activity of pig and rabbit CA III by reacting muscle carbonic anhydrase ($280\ \mu\text{M}$) with a 2-30 fold molar excess of phenyl glyoxal in 50 mM sodium diethylmalonate pH 7.0 at 30°C . Two hundred and eighty μM of enzyme is equivalent to $8.4\ \text{mg ml}^{-1}$, an unrealistically high working concentration. So, $34.6\ \mu\text{M}$ ($1.04\ \text{mg ml}^{-1}$) of chicken CA III and $110.3\ \mu\text{M}$ ($3.31\ \text{mg ml}^{-1}$) of human CA III were incubated with 1-50 mM phenyl glyoxal in 50 mM sodium diethylmalonate pH 7.0. The reaction mixture was incubated at 30°C in capped foil-wrapped tubes for 4-5 h. Aliquots were removed at intervals and assayed for bicarbonate dehydration and acid phosphatase activities.

2.12 COMPUTER ANALYSIS OF KINETIC DATA

All the kinetic data were analysed by 'Enzfitter' data analysis programme for the IBM PC based on non-linear regression. The Michealis-Menten equation was selected and all the points included with the simple weighting option. This sophisticated programme presented a graphic display of kinetic data, calculated V_{\max} and K_M with standard errors and also calculated the best fit Lineweaver-Burk plot. An example is included for reference (Figure 2.5 and Table 2.3).

TABLE 2.3 Computer Analysis of Kinetic Data

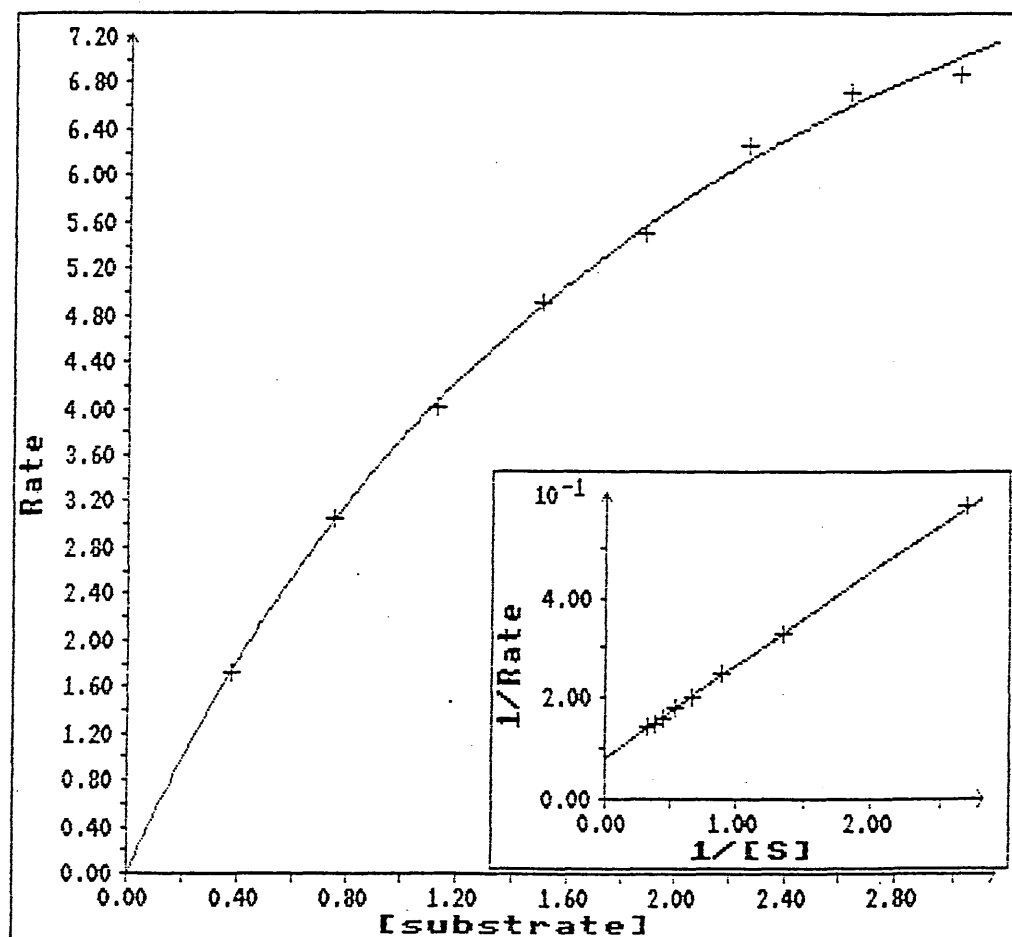
CA III chick -DPG

Michaelis Menten kinetics
Simple weighting

| Variable | Value | Std. Err. |
|----------|-------------|-------------|
| k cat | 1.24807E+01 | 4.93017E-01 |
| Km | 2.32718E+00 | 1.70683E-01 |

| | [substrate] | Rate | Calculated |
|---|-------------|-------------|-------------|
| 1 | 3.75000E-01 | 1.72000E+00 | 1.73203E+00 |
| 2 | 7.50000E-01 | 3.06000E+00 | 3.04191E+00 |
| 3 | 1.12500E+00 | 4.01000E+00 | 4.06721E+00 |
| 4 | 1.50000E+00 | 4.90000E+00 | 4.89159E+00 |
| 5 | 1.87500E+00 | 5.50000E+00 | 5.56883E+00 |
| 6 | 2.25000E+00 | 6.27000E+00 | 6.13511E+00 |
| 7 | 2.62500E+00 | 6.73000E+00 | 6.61562E+00 |
| 8 | 3.00000E+00 | 6.88000E+00 | 7.02848E+00 |

FIGURE 2.5 Computer Analysis of 4-NPA Hydrolysis Kinetic Data



Esterase activity chicken CA III (4-NPA hydrolysis). Control sample.

2.13 KINETIC MEASUREMENTS

Activity measurements, over a range of substrate concentrations, were conducted for the bicarbonate dehydration reaction, the hydrolysis of 4-NPA and HNTAS, and the acid phosphatase reaction using a number of CA isoenzymes from different sources. After computer analysis of this data, future research on activity measurements was carried out at $[S]$ well above K_M whenever possible. In some instances, however, due to the insolubility of the substrate or the high K_M which precluded accurate assays at high $[S]$, activity measurements were measured at $[S]$ below K_M and in excess of $[E_0]$. In these circumstances, the initial rate (v) is related to k_{cat}/K_M (refer to derivation) and may be represented by the apparent second order rate constant k_{enz} for a specified $[S]$.

Michaelis-Menten Equation

$$v = V_{max} [S]/(K_M + [S])$$

At low $[S]$, $[S] \ll K_M$

$$v = V_{max} [S]/K_M$$

Substituting $k_{cat} [E_0]$ for V_{max}

$$v = k_{cat} \cdot [E_0] \cdot [S]/K_M = k_{enz} [E_0] \cdot [S]$$

Despite the limitations discussed above and assuming that the chemical nature of the substrate was not appreciably altered by addition of components, since [S] is rate limiting, comparative studies were still valid and often reflect the 'in vivo' physiological milieu.

2.14 INORGANIC PHOSPHATE ASSAY (WATTS AND MORELAND, 1970)

Acid Molybdate Reagent

| | |
|---------------------|--------|
| Ammonium molybdate | 10 g |
| Distilled Water | 200 ml |
| 60% perchloric acid | 160 ml |

The above reagents were diluted to 1 litre.

Reducing Agent

| | |
|-----------------------|--------|
| Sodium metabisulphite | 12.0 g |
| Sodium sulphite | 4.8 g |

The sodium salts were dissolved in 50 ml distilled water, then added to 1-amino 2-naphthol 4-sulphonic acid (0.2 g) and diluted to 100 ml. After filtering twice, the reducing agent was stored in a dark bottle.

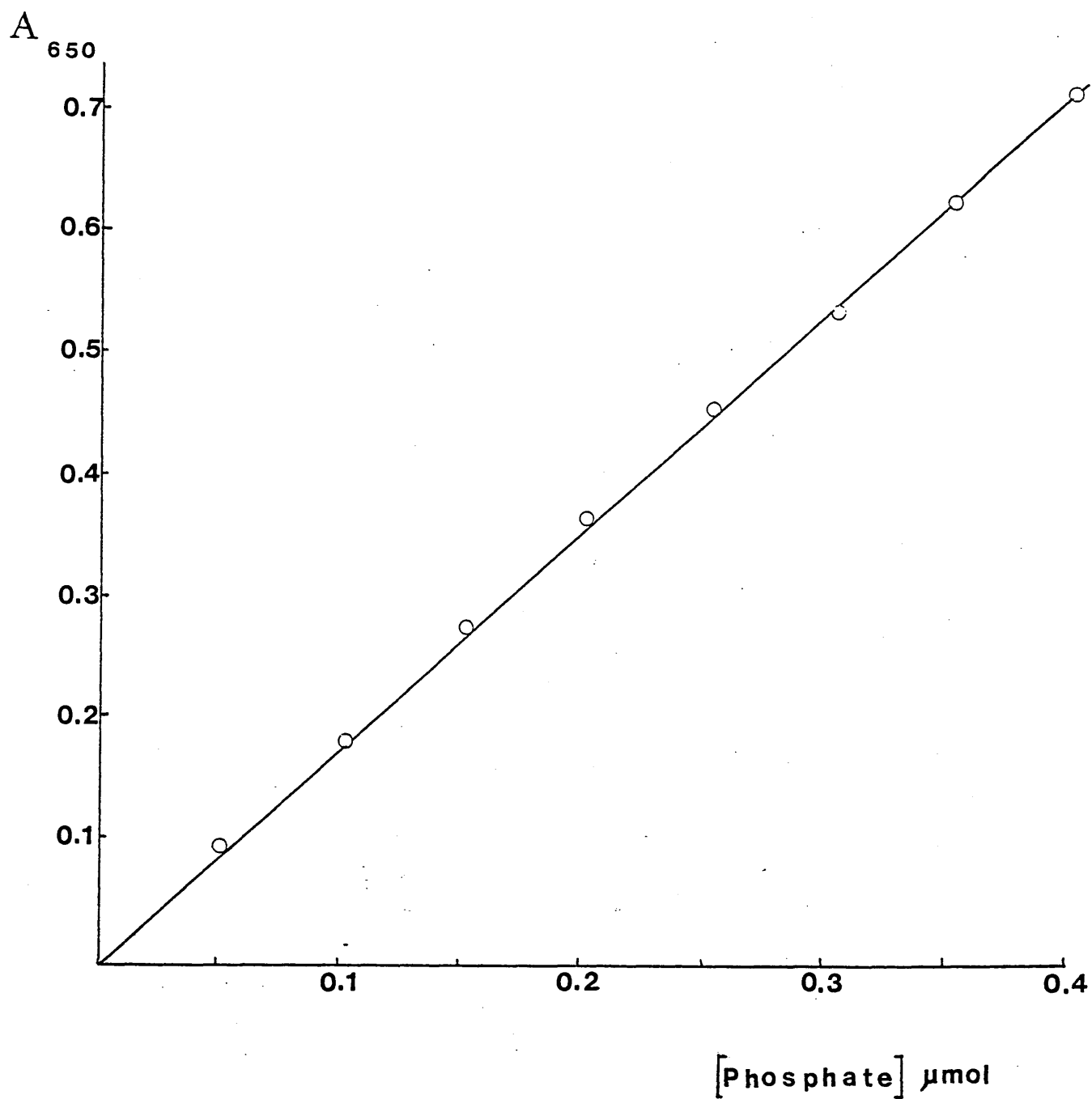
Standard Phosphate Solution

| | |
|--------------------------|-------|
| KH_2PO_4 | 1.7 g |
|--------------------------|-------|

After diluting to 250 ml, the standard phosphate solution was further diluted 1:50 with distilled water to give a final concentration of $1 \mu\text{mol ml}^{-1}$.

The assay was performed by adding 0.1 ml of acid molybdate reagent to 0.9 ml of test solution or standard phosphate. After shaking, 0.1 ml of reducing reagent was added (25°C). The absorbance was measured at 650 nm against a reagent blank after allowing 15 min for the colour to develop. A standard calibration curve was constructed using potassium dihydrogen phosphate (Figure 2.6).

FIGURE 2.6 Standard Curve for Determination of Inorganic Phosphate Concentration



2.15 PROTEIN ASSAY

The protein content of preparations from different tissues was determined to calculate the absorbance coefficients using the method of Ohnishi and Barr (1978). 3.2 ml of BS7 reagent was added to 0.8 ml of the sample (BS7 = Biuret reagent diluted 1:7 with 2.3% w/v sodium carbonate).

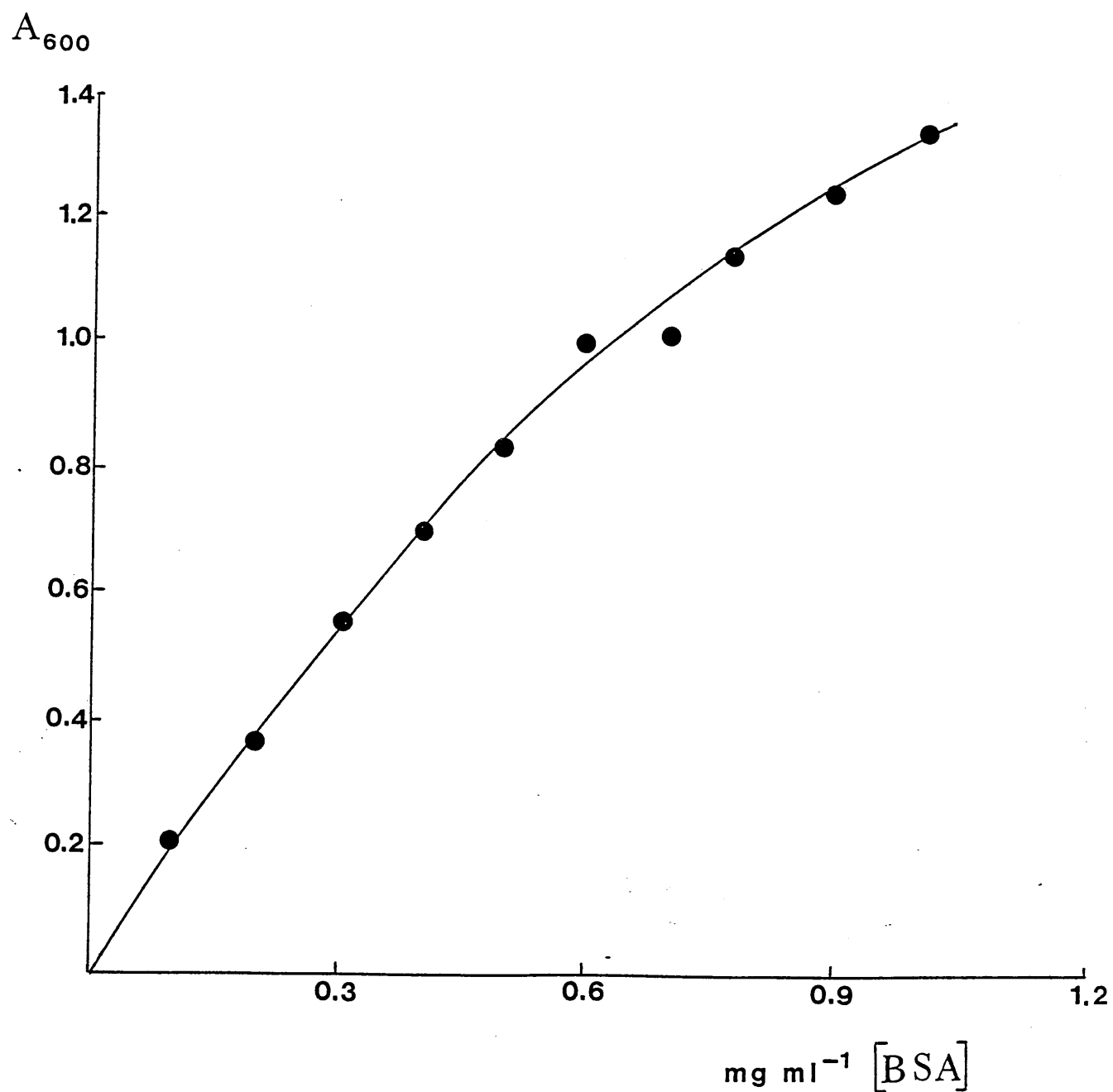
The solutions were mixed and left at room temperature for 10 min, then 0.1 ml of Folin and Ciocalteu phenol reagent was added and quickly mixed. Samples were incubated for a further 20 min and the absorbance measured against a water blank at 600 nm. A standard calibration curve was constructed using 0.1-1.0 mg ml⁻¹ bovine serum albumin (Figure 2.7).

Absorbance coefficients for CA samples of known protein content were calculated by measuring the absorbance at 280 nm.

Absorbance Coefficients

| | |
|----------------|---|
| Chicken CA III | $1.21 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ |
| Human Ca III | $1.80 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ |
| Horse CA III | $1.88 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ |
| Frog Protein | $0.96 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ |

FIGURE 2.7 Standard Curve for the Determination of Protein Concentration



2.16 DETERMINATION OF RELATIVE MOLECULAR MASS

The relative molecular masses of unknown proteins were determined by comparison with markers of known RMM on SDS-PAGE and by gel filtration. The migration distance from the stacking gel/separation gel interphase on SDS-PAGE was measured for: Bovine serum albumin (67,000), carbonic anhydrase (29,000) and myoglobin (17,600). A plot of the logarithm base 10 of the RMM against migration distance yields a straight line, and the approximate RMM of any unknown band can thus be calculated.

A similar method was employed using a gel filtration column. Again a straight line was obtained by plotting the natural logarithm of the RMM's of known protein markers against fraction number from a calibrated column. The myoglobin peak was pinpointed by measuring the absorbance at 410 nm, the CA peak by the BTB spot test and measuring the absorbance at 280 nm and the bovine serum albumin peak by measuring the absorbance at 280 nm.

2.17 MATERIALS AND SUPPLIERS

(1) Gases

Carbon dioxide and nitrogen gas filled cylinders:

British Oxygen Company

Leeds

West Yorkshire

(2) Enzymes and Reagents

(a) Carbonic anhydrase:

Sigma Chemical Co

Fancy Road

Poole, Dorset

BH17 7NH

(b) Column chromatography reagents:

G75 Sephadex, Bead size 40-120 μm and Blue Dextran:

Sigma Chemical Co

DEAE Tris acryl M and 100 HR Sephacryl:

Pharmacia Ltd

Pharmacia House

Midsummer Boulevard

Milton Keynes

Bucks

MK9 3HP

(c) Electrophoresis reagents:

Coomassie Blue and Agarose Type II Medium EEO (0.16-0.19):

Sigma Chemical Co

Sodium Dodecyl Sulphate, extra pure grade:

BDH

Broom Road

Poole

Dorset

BH12 4NN

(d) Enzyme assay reagents:

Bromothymol Blue, pH 6.0-7.6:

BDH Ltd

Acetazolamide:

Sigma Chemical Co

Ethoxzolamide (now discontinued):

Sigma Chemical Co

Acetone (AR):

BDH Ltd

**3 STUDIES OF MUSCLE CARBONIC ANHYDRASE AND
ASSOCIATED PROTEINS DURING ISOLATION.**

3 STUDIES OF MUSCLE CARBONIC ANHYDRASE AND ASSOCIATED PROTEINS DURING ISOLATION

3.1 INTRODUCTION

Whilst isolating muscle carbonic anhydrase a number of observations and anomalies were noted, which prompted an investigation into proteins 'associated' with carbonic anhydrase during the extraction procedure. These observations included the regular appearance during purification of the same minor contaminating band on SDS-PAGE of chicken CA III and the visualisation of an early peak of BTB-positive samples from the affinity resin and occasionally from the G75 gel filtration column. On occasions chicken CA III samples produced anomalous esterase kinetic data and in many pure CA III preparations there was no appreciable acid phosphatase activity.

The intriguing possibility that one or more carbonic anhydrase 'associated' proteins may infrequently affect or contribute to activity measurements could not be overlooked. Consequently, several preparations of chicken CA III and frog muscle protein were examined more fully, with particular emphasis on fractions that would under normal circumstances be discarded. A limited investigation was undertaken to characterise the physical and chemical properties of these muscle 'associated' proteins.

3.2 STUDIES OF CHICKEN CA III-ASSOCIATED PROTEINS

3.2.1 SDS-PAGE of Chicken Muscle CA-associated Proteins

The method of extraction of chicken CA III was as described in section 2.1. After sodium sulphate gradient elution from the affinity resin, fractions from the beginning (18), middle (25) and end (35) of the BTB-positive peak were subjected to SDS-PAGE. The array of contaminating proteins are shown in Plate 3.1 (lanes 10, 9 and 8 respectively) together with a chicken CA III sample purified to homogeneity.

The predominating protein in fraction 18 (lane 10) is not carbonic anhydrase, but a protein with a RMM of \approx 41,000. This band designated protein A, for convenience, is also evident in fraction 25 (lane 9) at a relatively high concentration as compared to CA III. It diminishes in proportion by the end of the BTB active peak. Thus, at the centre of the CA peak, as determined by the BTB spot test, a significant concentration of protein A is present as a contaminating factor.

A fraction (17) containing a high proportion of protein A was subsequently found at a lower loading to comprise two bands (lane 6) with approximate molecular masses of 31,000 and 41,000. Further study of this protein A-rich fraction proved difficult because of its rapid precipitation at 4°C.

Another significant band eluted at a higher salt concentration was visualised in fractions 25 and 35 (lanes 9 and 8) above protein A. This protein was designated protein B.

(e) Phosphorylated metabolites were all obtained from
Sigma Chemical Co

ATP Disodium salt 99% - 100% grade

D(-) 3-phosphoglyceric acid, sodium salt Grade I

2,3-diphosphoglyceric acid, Tris salt

phosphoenolpyruvic acid, trisodium salt

(f) Frozen tissues:

Frog's legs:

Mr M Holdsworth Ltd

Buxton

(Tel 0298 871435)

Chicken thighs:

J Sainsbury's plc

All other chemicals were analytical grade and obtained from
either Sigma Chemical Co or BDH Ltd.

BSA

protein B
protein A
CA

CA

myo-
globin

1 2 3 4 5 6 7 8 9 10 11 12

- 1 Markers. BSA, CA, myoglobin.
- 2 Chicken CA III
- 3 Human CA III (polymorphic subject)
- 4 Markers + protein A (fraction 17)
- 5 Protein A (17) High loading
- 6 Protein A (17) Low loading
- 7 Markers
- 8 Fraction 35 (end affinity resin BTB + ve peak)
- 9 Fraction 25 (middle affinity resin BTB + ve peak)
- 10 Fraction 18 (beginning affinity resin BTB + ve peak)
- 11 Human CA I
- 12 Chicken CA III

PLATE 3.1 SDS-PAGE of Chicken CA III and Associated Proteins Eluted
from Sulphonamide Affinity Resin

3.2.2 Anodic DISC-PAGE

Proteins A and B were analysed by DISC-PAGE to determine their relative charge in the native state. Protein A failed to migrate, or only just entered the stacking gel, whilst protein B migrated towards the anode, albeit a short distance (Plate 3.2). Chicken CA III, a basic protein (Holmes, 1977), carries insufficient negative charge to enter this gel designed to separate acidic and neutral proteins.

Chicken CA III was added to proteins A and B contained in fraction 25 in the proportions 1:3, in an attempt to establish any possible link between them. No additional acidic or neutral band was evident. A complex with a basic charge and zero migration under these conditions cannot however be ruled out.

r " " fft
6 ----
6

8SA* 6SA

cA*L cAH

■# 8>SA MyO SSA ***
CO#^aM&Auctr

1 2 3 4 5

Lanes

- 1 Myoglobin
- 2 BSA + chicken protein B + human CA II
- 3 BSA + human CA II
- 4 Chicken proteins A and B
- 5 Human CA I

PLATE 3.2 Anodic DISC-PAGE of Native Chicken Proteins A and B

3.2.3 Cathodic DISC-PAGE

Proteins A and B failed to migrate on a cathodic DISC-PAGE aimed at separating basic proteins.

3.2.4 Ion-exchange Chromatography and Gel Filtration of CA III and 'associated' Proteins

Since protein A carries almost no negative charge, whereas protein B is slightly acidic, then anion ion-exchange chromatography of pooled fractions 18-35 removed none of the protein A component but a considerable proportion of protein B. Thus, after ion-exchange chromatography and vacuum dialysis, essentially only two proteins (CA and protein A) were loaded onto a G75 gel filtration column. Two BTB-positive peaks emerged and are shown in Figure 3.1. The first peak corresponding to a RMM of $\approx 70,000$ or more, contained the major proportion of protein. Though smaller in protein content, the second peak with CA activity corresponded to the molecular mass of carbonic anhydrase (29,000).

A fraction from the centre of each BTB-positive peak was analysed by SDS-PAGE (fractions 8 and 32). It is evident from Plate 3.3 that the protein A-rich sample (8) in lane 1 is devoid of a carbonic anhydrase band despite the positive BTB test. Two bands are present in this sample with approximate RMM values of 41,000 and 38,000. It may well be that protein A visualised as one band on cruder preparations is in fact a dimer eluted from the G75 gel filtration column at 70 kD or more, or two proteins of similar RMM in a single complex. Breakdown products of this dimer complex can conceivably contaminate CA as shown in the impure CA III sample in

lane 6. This poor CA sample also contains some myoglobin, although protein B appears to be absent. It emphasises the necessity of discarding fractions at each end of the BTB-positive peak and careful monitoring of new preparations. Extractions containing an initial high concentration of protein A would be likely to contain some contaminating factor(s) at the final stage and would thus be unsuitable for precise kinetic studies.

Protein A fraction 25 (see Figure 3.1)

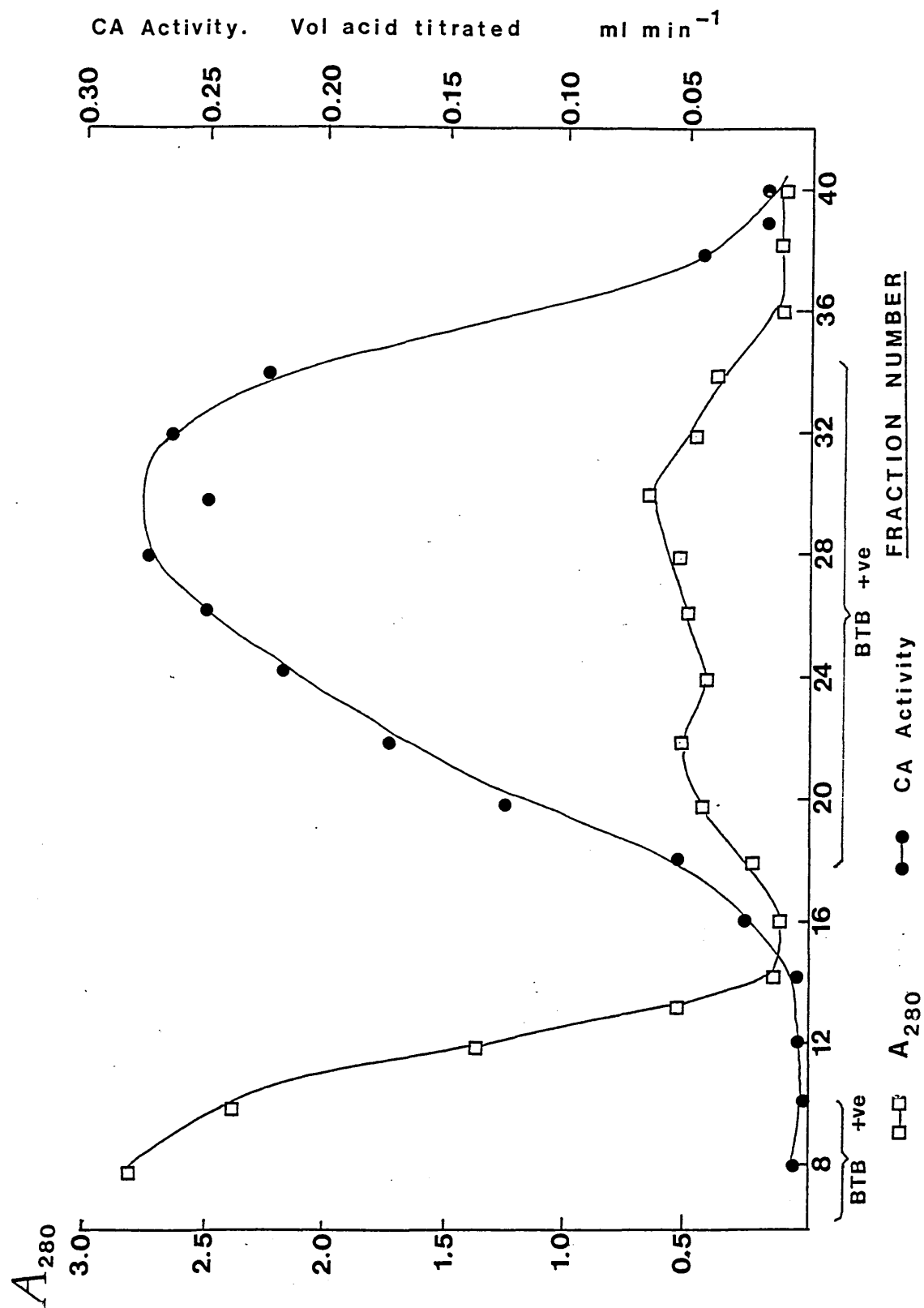
Crude homogenate with predominance protein A
Pure chicken CA III (fraction 32 from G75 column)
Human CA I
Impure chicken CA III preparation

1 2 3 4 5 6 7 8

- 1 / 7 / 8 Protein A fraction 8 (see Figure 3.1)
- 2 Affinity resin fraction 25 containing protein A and CA III
- 3 Crude homogenate with predominance protein A
- 4 Pure chicken CA III (fraction 32 from G75 column)
- 5 Human CA I
- 6 Impure chicken CA III preparation

PLATE 3.3 SDS-PAGE Analysis of Protein A from the Gel Filtration Column

FIGURE 3.1 Analysis of Chicken CA III Preparation Fractions by Gel Filtration Chromatography



3.2.5 Activity Measurements of Chicken CA-associated Proteins

a) Bicarbonate Dehydration Activity

Prompt pH-stat bicarbonate dehydration assays were performed on a protein A-rich fraction (8) and a CA-rich fraction (32) straight from the sephadex column before any precipitation could occur in the former sample. As expected, carbonic anhydrase activity was detected in fraction 32, but no activity was evident in fraction 8.

b) 4-NPA Esterase Activity

Esterase activity was assayed in fraction 8 containing essentially only protein A and a significant 4-NPA hydrolysis was monitored. Unfortunately, accurate protein determinations were not taken before some precipitation occurred, which precluded specific activity calculations. An approximation of protein content based on an absorbance of 2.9 at 280 nm, indicated that the specific activity was not in excess of the level exhibited by chicken CA III. Results from the gel filtration column indicate that protein A exists as a protein/complex of $RMM \approx 70,000$, which on reduction yields two bands of $RMM \approx 41,000$ and $38,000$. Presumably, the esterase activity is possessed by protein A in its complexed dimeric form. In order to exclude any involvement of protein A subunits in the esterase activity of an impure chicken CA III preparation, it would be necessary to separate the $41,000$ and $38,000$ subunits from CA and determine any possible activities.

3.3 STUDIES OF FROG MUSCLE PROTEINS

A pilot study on the purification of amphibian muscle carbonic anhydrase in this laboratory some time ago, resulted in the isolation of an esterase. This esterase possessed relatively little bicarbonate dehydration activity, but it did remain in solution at 4°C. In view of recent data recorded in section 3.2, this exercise was repeated and the results are presented below.

3.3.1 SDS-PAGE of Frog Muscle Proteins

Two preparations were completed using essentially the same extraction method as for chicken CA III. BTB spot testing of fractions collected from the affinity resin yielded six slightly positive samples, each of which contained two bands on SDS-PAGE gels. These bands with approximate RMM's of 42 kD and 38 kD are comparable to protein A from chicken muscle. No frog muscle carbonic anhydrase was evident. However, SDS-PAGE analysis of the second preparation using an increased quantity of frog muscle revealed the existence of a band corresponding to CA. The relative concentration of this 'CA' band was small by comparison to frog protein A. Analysis of this sample using a lower loading also showed the existence of a band with a RMM of \approx 47 kD comparable to chicken protein B.

Thus, notwithstanding the evolutionary diversity of birds and amphibians, it is conceivable that data compiled for these frog proteins can be related to the chicken data.

3.3.2 Anodic and Cathodic DISC-PAGE

Frog muscle proteins were electrophoretically similar to chicken A and B proteins, in that they failed to migrate to the anode or only scarcely entered the gel on anodic PAGE. They were not visualised on a cathodic polyacrylamide gel.

3.3.3 Flat-bed Electrophoresis and the Effect of Dansylamide

The method developed in this laboratory for the analysis of neutral/basic proteins under non-reducing conditions, described in section 2.7, was applied to frog preparation 1 after storage at -70°C for 12 weeks and fresh frog preparation 2. Both were subjected to electrophoresis with and without dansylamide (Plates 3.4 and 3.5). Whilst no discrete fluorescent bands were visible, frog preparation 2 was completely immobilised by this sulphonamide as visualised after Coomassie Blue staining. The migration of the frog sample subjected to freezing was only impeded by 20% and that of CA I by 10%.

Whether or not the reduced response of preparation 1 to dansylamide as compared to sample 2 was due to the length of storage of this sample, the apparent presence of a B-type protein or another factor is impossible to determine. Whatever the reason, the reduced migration of both these samples with dansylamide does demonstrate the affinity that these contaminating proteins must have for this particular sulphonamide.

©

©

CA X

CA 1 +
3rJSA

Ervj

~~DrSA~~

PLATE 3.4 Electrophoresis of CA I and Frog Preparation 1 and the Effect of Dansylamide

○

©

2

€

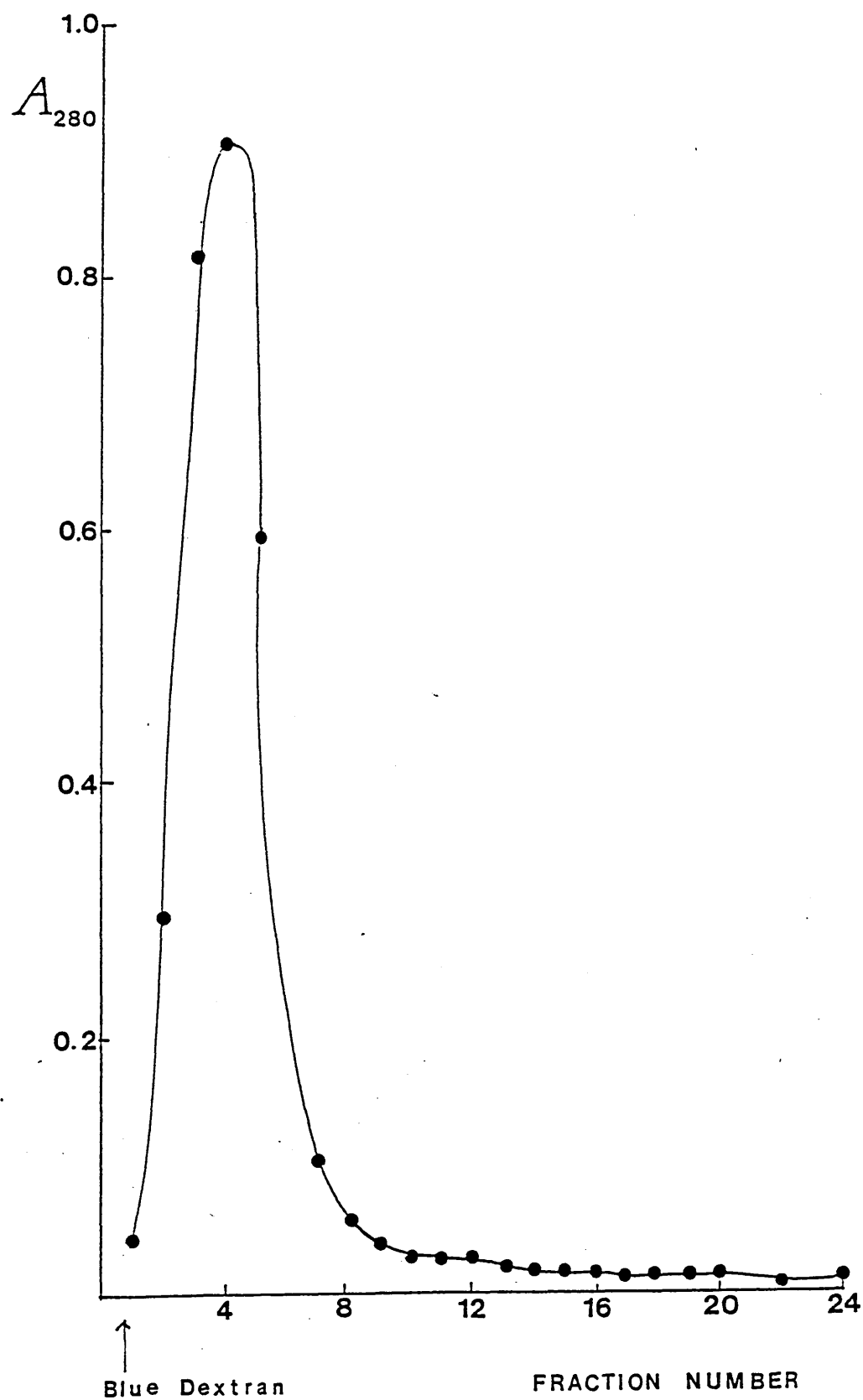
x -t-

PLATE 3.5 Electrophoresis of Frog Muscle Preparation 2 and the Effect of Dansylamide

3.3.4 Gel Filtration

Figure 3.2 shows the profile of protein elution from a G75 sephadex column of the pooled, vacuum dialysed BTB 'active' peak of frog preparation 2. It is clear that almost all the protein was eluted immediately after the blue dextran. This indicates the possible dimeric quaternary structure of the apparent type A frog protein. Calibration of this column indicated the corresponding RMM to be around 70,000 or more (Figure 3.3). No carbonic anhydrase peak is evident. Any interference by blue dextran was excluded, by replacing this marker with myoglobin (absorbance 410 nm), an approximate RMM of 70,000 or more was again observed.

FIGURE 3.2 Determination of the Relative Molecular Mass of Frog Protein A by G75 Gel Filtration Chromatography.



Rea.k. C ^zv©')

3.X

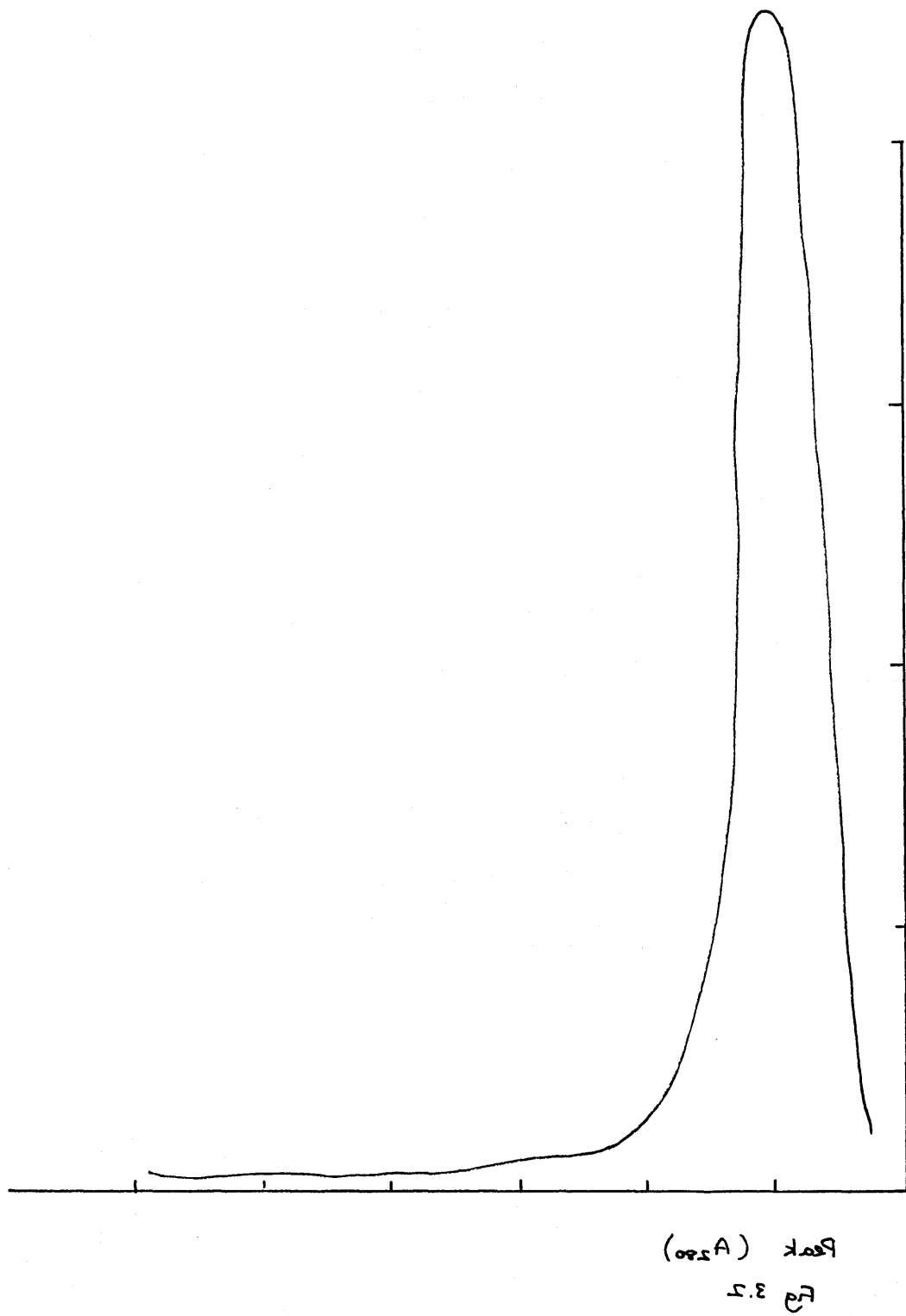
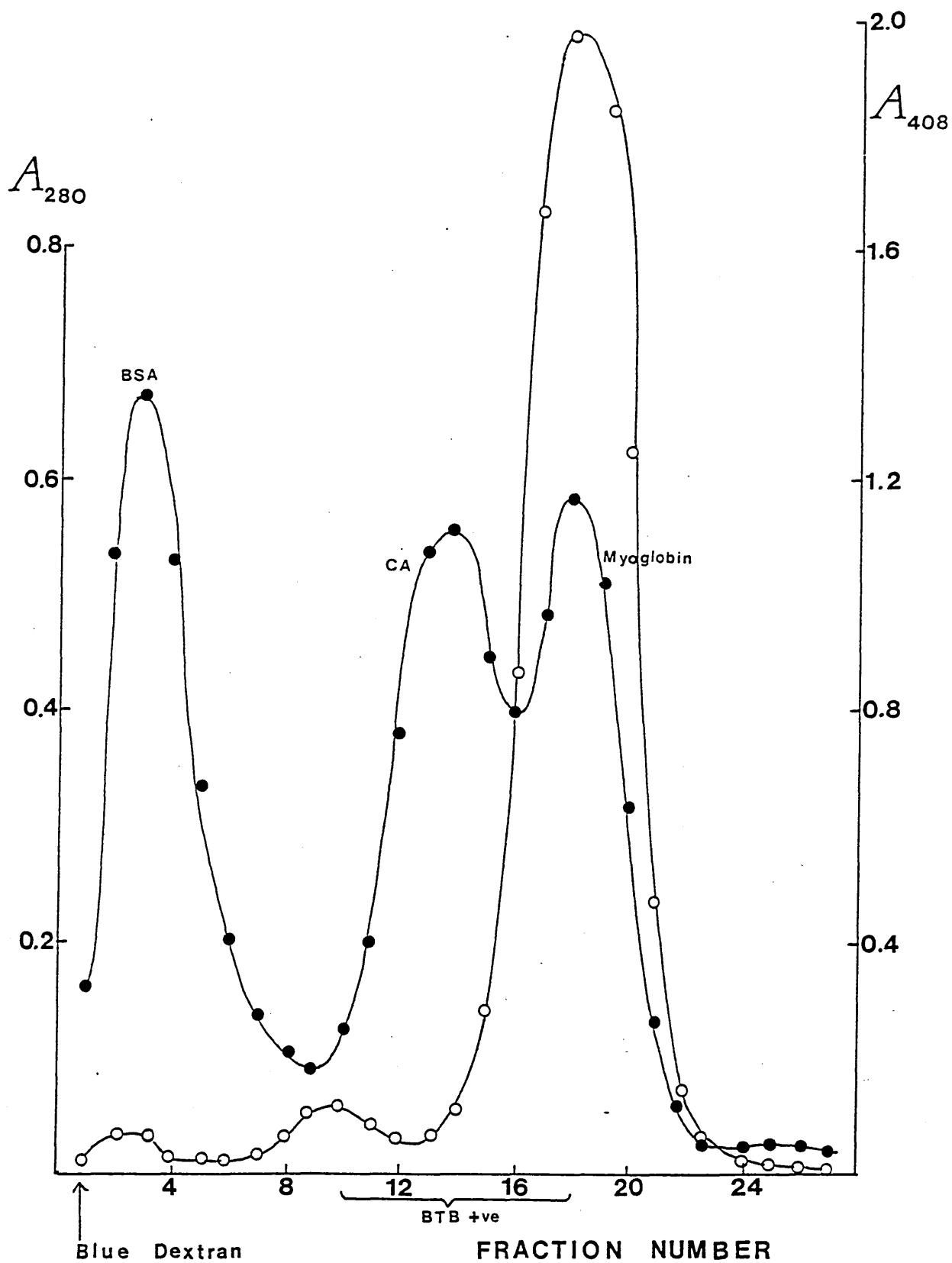


FIGURE 3.3 Calibration of a G75 Sephadex Column



(●—●) A_{280} ; (○—○) A_{408}

0.5 ml containing 0.05 mg ml^{-1} bovine serum albumin, bovine carbonic anhydrase and myoglobin was loaded onto a G75 Sephadex column (100 x 1.5 cm) at 40 ml h^{-1} . 4 ml fractions were collected.

3.3.5 Activity Measurements of Frog Proteins

a) Bicarbonate Dehydration Activity

No bicarbonate activity was demonstrated in any of the frog muscle preparations by pH-stat assay, and in fact the BTB spot test activity declined after a week's storage of the sample. The initial positivity of these early samples is difficult to adequately explain. A small CA band was demonstrated on SDS-PAGE by sample overloading in the second preparation, but this was not evident in the first sample and both yielded a positive spot test. The possibility that CA is complexed to the type A protein cannot be ruled out, although drastic reducing treatment followed by SDS-PAGE did not reveal a CA band.

Examination of the pH of the BTB positive samples suggested some acidification from 8.7 to 8.3, hardly sufficient to mediate a blue to yellow colour change.

Inclusion of 0.01 M acetazolamide in the bromothymol blue test solution did not diminish the development of a yellow spot in the newly prepared frog extraction. This again tends to exclude involvement by carbonic anhydrase.

b) Esterase Activity

Frog preparation 2 containing predominantly an apparent type A protein and some type B protein also exhibited esterase activity towards 4-NPA, in accordance with the data presented for chicken A protein (3.2.5. b). The preparation which had been frozen also retained some esterase activity.

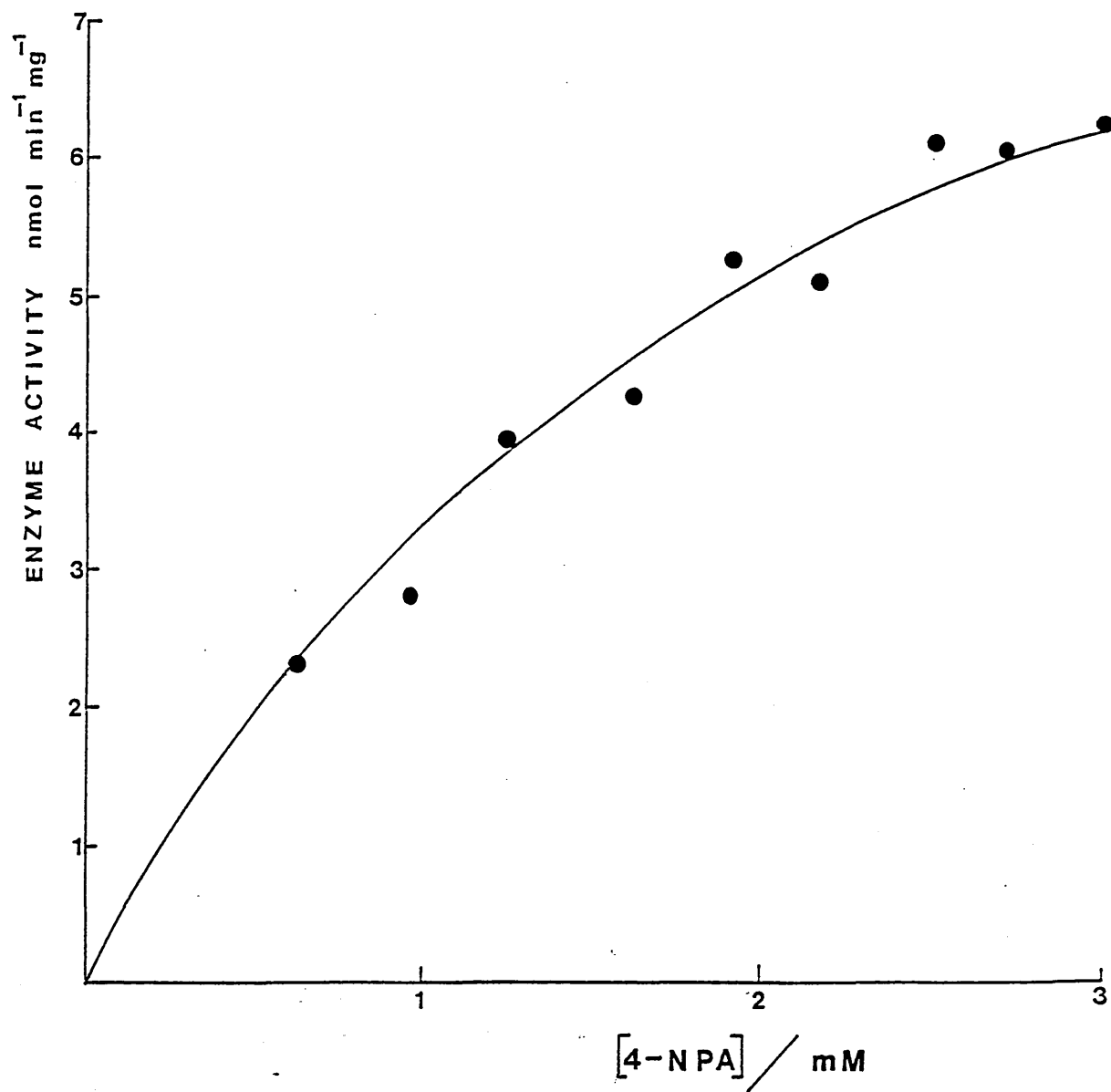
The stability and apparent solubility of the frog protein(s) permitted a limited study of the kinetic and inhibition parameters for the esterase reaction (Table 3.1). The kinetic data for the frog protein(s) are shown in Figure 3.4. In order to determine the absorbance coefficient of the frog protein ($0.96 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$), it was assumed that the majority of protein was type A. A comparison of the kinetic parameters of this frog protein(s) with chicken CA III in Table 3.1 shows that they are of the same order of magnitude.

TABLE 3.1 Kinetic Parameters of Chicken CA III and Frog Protein for 4-NPA Hydrolysis

| | K_M mM | V_{max} nmol min ⁻¹ mg ⁻¹ |
|----------------|-------------|--|
| Chicken CA III | 1.7 | 12.5 |
| Frog Protein | 3.1 | 13.0 |

Surprisingly, no inhibition of the esterase activity was evident with 1.25 mM acetazolamide or 0.125 M chloride, and only 15% inhibition was demonstrated with 0.0375 mM ethoxyzolamide, a potent inhibitor of the esterase activity of CA I and CA II.

FIGURE 3.4 Michaelis-Menten Curve for the Hydrolysis of 4-NPA by Frog Muscle Protein



c) Acid Phosphatase Activity

Koester et al (1981) demonstrated a low acid phosphatase activity in rabbit CA III as a unique characteristic of the type III isoenzyme. In contrast, Nishita and Deutsch (1986) reported acid phosphatase activity in equine CA I, II and III but at even lower levels.

Studies in this laboratory suggest that some chicken CA III and human CA III preparations possess a low acid phosphatase activity, but in agreement with Deutsch this activity is not confined to the muscle isoenzymes (Table 3.2). Human CA I also had a measurable phosphomonoesterase activity.

In many preparations of chicken and human CA III, however, the level of activity was negligibly low and it is worth noting that acid phosphatase activity was also demonstrated in chicken protein A sample containing virtually no CA and in frog preparation 2. The negative human and chicken preparations were exceptionally pure samples and the assays were repeated several times with the appropriate controls.

Activity measurements reported from other species are included in Table 3.2. Assuming a 5% contamination by an equivalent protein A in the muscle extractions, it is not inconceivable that the low levels of reported activity could be accounted for by such a contaminating factor.

Contrary to inhibition studies by Koester et al (1981), phenyl glyoxal and phosphate did not inhibit acid phosphatase activity of chicken CA III.

TABLE 3.2 Acid 4-nitrophenyl Phosphatase Activity of CA III and Associated Proteins

| ENZYME | INHIBITOR/MODIFIER | SPECIFIC ACTIVITY nmol min ⁻¹ mg ⁻¹ |
|-------------------|----------------------|--|
| Chicken CA III | | 0.063 |
| | 12 mM phenyl glyoxal | 0.059 |
| | 42 mM phosphate | 0.062 |
| Human CA I | | 0.045 |
| Human CA III | | 0.061 |
| Chicken protein A | | 0.024 |
| Frog prep 2 | | 0.071 |
| Rabbit CA III* | | 0.47 |
| Horse CA III** | | 0.018 |
| Horse CA II** | | 0.029 |
| Horse CA I** | | 0.04 |
| Bovine CA III* | | 0.095 |

Koester et al* (1981)
Nishita & Deutsch** (1986)

3.4 DISCUSSION

The compilation of data in this section is important for three reasons. Firstly, it allays any misgivings that precise chicken CA III activity measurements recorded in sections four and five, might be distorted by the presence of contaminating proteins, because only exceptionally pure samples were used. Secondly, it provides valuable information about the nature of proteins associated with muscle CA during isolation and the necessity of rigid laboratory practice, and thirdly it raises the vexing question of whether these associated proteins are specifically or non-specifically linked to muscle CA metabolism. The first two points will be discussed in detail in this section, whilst the last issue will be considered in the final discussion.

Undoubtedly, carbonic anhydrase is not the only muscle protein with a strong affinity for $p[(2,4\text{-diaminophenyl})\text{azo}]$ benzenesulphonamide. Moreover, it is not the only muscle protein that in addition to binding to prontosil, carries a positive charge and possesses esterase activity towards 4-NPA and some acid phosphatase activity.

It is clear from photographs of SDS-PAGE that by employing the methods described in this thesis, pure homogeneous preparations of CA III can be prepared, but it is also clear that the purifier needs to be aware of the existence of other molecules that share certain common properties with carbonic anhydrase. Analysis of the active fractions of affinity resin salt elution from chicken muscle demonstrates the presence of a protein (A), which binds firmly to the prontosil and is eluted at a slightly lower salt concentration than CA, but which nonetheless overlaps with the CA peak. It is thus

impossible in the early stages of muscle CA preparation to eliminate this protein.

Limited characterisation of this chicken protein, which has a low aqueous solubility, indicates that it may be a dimer, comprising two sub-units of RMM \approx 41,000 and 38,000. Its basic/neutral nature, in common with chicken CA III, probably contributes to the similar elution pattern of these two proteins on anion exchange chromatography.

A second impurity (protein B), eluted at a slightly higher ionic strength than chicken CA III, bears a small negative charge and has a RMM of \approx 45,000.

By following the course of these two proteins it is apparent that this second protein is almost entirely removed by anion exchange chromatography and the possible dimer is usually effectively separated on the basis of molecular mass by gel filtration. Nevertheless, whether by over zealous collection of BTB active fractions or possibly by the formation of complexes, some chicken preparations are contaminated by protein A, as exemplified by SDS-PAGE. Whether these impure preparations contain the dimer/complex or its sub-units is impossible to state; after gel filtration monomer contamination would seem most plausible.

Based on the consideration that a chicken preparation might contain say 10% contamination with this protein A, would it contribute wholly or partially to activity measurements? The method adopted for CA III purification is one in current use, and many researchers quote "that one major band corresponding to a molecular weight of 29,000 is present on SDS-PAGE." This in itself implies that there are minor ones (Tu et al, 1986). In an attempt to try and answer this question

various activity measurements were conducted with the primary CA-associated protein A.

No bicarbonate dehydration activity was detected using the pH-stat assay system in dimer/complex-rich fractions, despite the fact that these samples were initially positive in the BTB spot test for CO₂ hydration. No CA band has ever been visualised on SDS-PAGE in these early BTB-positive fractions from the sephadex column and the addition of acetazolamide at concentrations that would normally inhibit CA activity has no effect on the spot test. Thus, it seems unlikely that these samples produce positive spot tests because of the presence of carbonic anhydrase. Moreover, the addition of protein A, even at milligram concentrations, to CA active fractions did not affect bicarbonate dehydration activity. Therefore, it is probable that even 10% contamination by protein A would have little effect on bicarbonate dehydration activity.

The demonstration that chicken protein A has esterase activity towards 4-NPA is interesting and it was imperative to determine the specific activity of this possible dimer and to quantify its kinetic parameters. Owing to the low solubility of the chicken dimer/complex, attention was turned to the apparently equivalent, more soluble frog protein, which displays similar chemical and physical properties to the chicken CA-associated protein A. It is probably dimeric in structure with subunits of $\approx 42,000$ and $\approx 39,000$, carries a positive charge and has a strong affinity for dansylamide, as demonstrated by total immobility on flat-bed electrophoresis with this sulphonamide. In accordance with the chicken data it hydrolyses 4-NPA and possesses phosphomonoesterase activity but no bicarbonate dehydration activity.

The frog muscle protein exhibited very similar kinetic properties to chicken CA III with similar K_M values for 4-NPA.

Although comparisons between species are not entirely valid it is not improbable that contamination with an equivalent protein might indeed, even at 10%, produce spurious kinetic data. These studies highlight the need for meticulous CA III preparation particularly for esterase activity measurements.

Acid phosphatase specific activity measurements of chicken protein A and the apparently equivalent frog muscle protein were also of the same order of magnitude as those for chicken CA III. A 10% contamination of chicken CA III by protein A would not therefore account for all the acid phosphatase activity. However, the absorbance changes were exceptionally low and many pure samples of chicken CA III and human CA III exhibited no acid phosphatase activity at all. Only one chicken and one frog type A protein was examined, whilst many CA III samples were analysed. It is not inconceivable that the acid phosphatase activity demonstrated could be accounted for by this 'contaminating' protein. Indeed, comparison of levels of activity reported from some other laboratories with those demonstrated by these CA-associated proteins would wholly contribute to activity measurements.

The results presented in this thesis indicate that, in agreement with Nishita and Deutsch, acid phosphatase activity is not confined to the type III muscle isozymes, since some activity was demonstrated in human CA I. In fact, the results indicate that this area is not clear cut and that the statement "muscle CA III possesses a unique acid phosphatase activity" is far from proven.

In summary, it is apparent from this data that binding to sulphonamide affinity resin is not a specific property of the carbonic anhydrases and that, without due care, the non-specifically bound proteins may be retained throughout the CA III extraction procedure.

Although not detrimental to bicarbonate dehydration activity measurements, it is likely that contamination by one of these proteins can result in spurious esterase data and may wholly contribute to acid phosphatase measurements.

It is worth noting, that many physiological experiments with whole muscle, are based on the assumption that the carbonic anhydrases are the only major proteins responsive to sulphonamide inhibition at the concentrations employed. Although 4-NPA esterase activity was not inhibited by acetazolamide or chloride in the frog protein sample, binding of dansylamide profoundly affected electrophoretic migration. It is not improbable that some sulphonamides could severely curtail any in vivo physiological function of this muscle CA-associated protein, and perhaps contribute to muscle performance.

4 BICARBONATE DEHYDRATION ACTIVITY AND ACTIVE SITE STUDIES

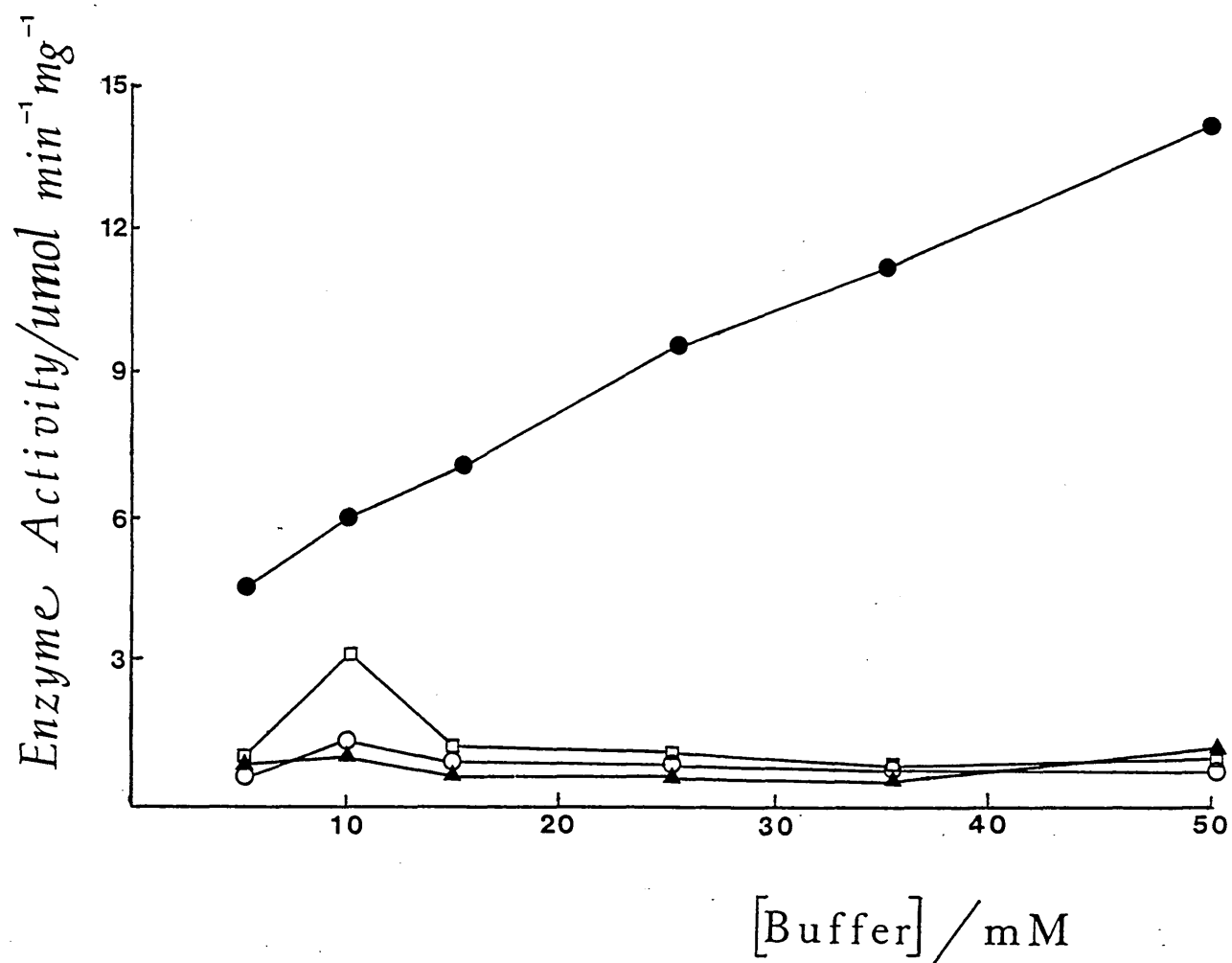
4.1 INTRODUCTION

An investigation of the bicarbonate dehydration activity of CA III was undertaken to characterise this particular isoenzyme, to relate these results to active site residues unique to CA III and to compare and contrast its properties with those of other isoenzymes in the multigene family. Firstly, an appropriate inert buffer was selected in order to investigate the possible effects of various metabolites and ions. These effects were monitored by changes in specific activity measurements and whenever feasible they were characterised by determination of the kinetic parameters. The specific nature of the effects was investigated by active site modification and by more extensive study.

4.2 SELECTION OF AN 'INERT' BUFFER

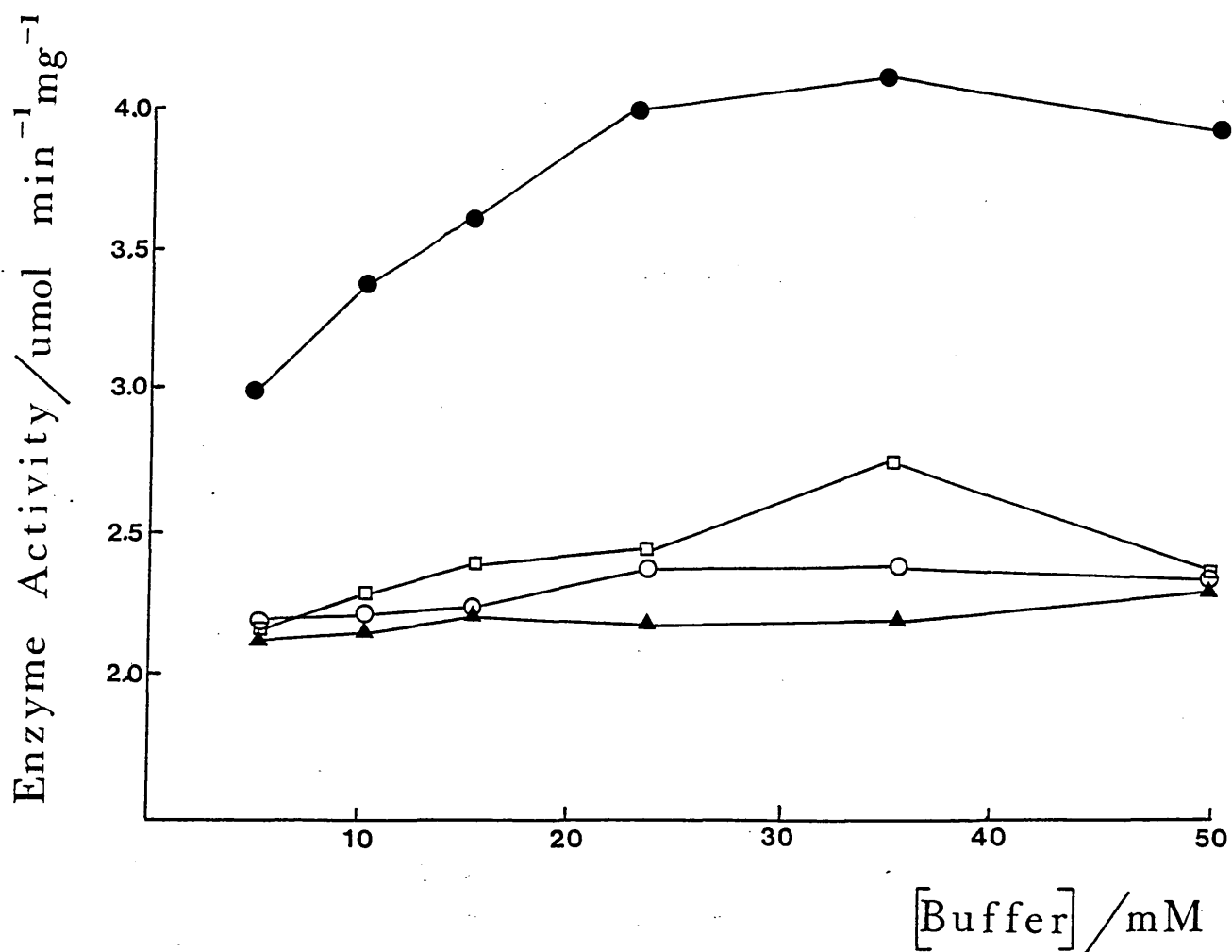
The bicarbonate dehydration activities of chicken CA III and human CA III were determined in four buffers, namely hepes, pipes, mops and phosphate, to ensure that the assay buffer would not contribute to any activity change. Buffer concentration ranged from 5.0 to 50.0 mM. The buffering capacity below 5.0 mM was insufficient to provide smooth traces. Figures 4.1 and 4.2 show that for both chicken and human CA III, a marked enhancement of activity occurred in phosphate buffer, activation being greater in the avian preparation (Shelton and Chegwidde, 1988). In contrast, the other three buffers were essentially without effect and in subsequent investigations, hepes, at a final concentration of 10 mM, was selected as an ideal buffering system.

FIGURE 4.1 The Effects of Different Buffers on the Bicarbonate Dehydration Activity of Chicken CA III.



Bicarbonate dehydration activity measurements were made in 30 mM NaHCO₃, pH 7.1, 2°C. (●-●) phosphate; (▲-▲) hepes; (□-□) pipes, (○-○) mops.

FIGURE 4.2 The Effects of Different Buffers on the Bicarbonate Dehydration Activity of Human CA III.



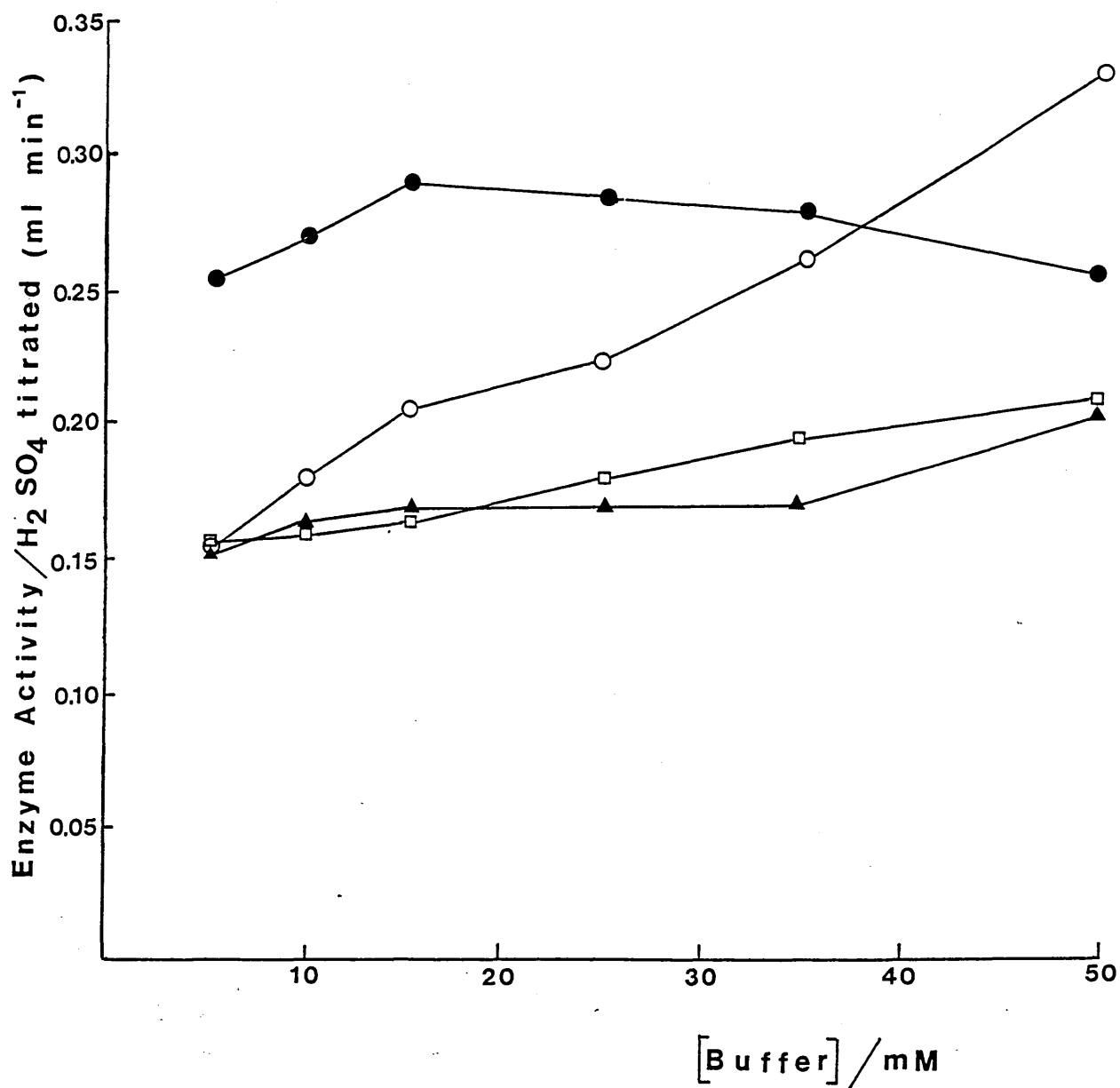
Bicarbonate dehydration activity measurements were performed in 30 mM HCO_3^- , pH 7.1, 2°C. (●-●) phosphate; (▲-▲) hepes; (□-□) pipes; (○-○) mops.

The addition of phosphate to assays already containing hepes resulted in an equivalent degree of activation, dependent on concentration. It is worth noting that, below 5.0 mM, phosphate with 10.0 mM hepes was without effect. Thus phosphate activation was not simply an ionic strength effect; a premise substantiated by a control experiment in which the activity remained constant with increasing glutamate concentration. The use of simpler ions, such as sulphate and chloride was avoided because of their inhibitory properties. A supplementary study, with phosphate at a constant 10.0 mM and increasing hepes buffer (5.0-50.0 mM), showed that the percentage activation by phosphate remained essentially constant at 200-300%.

The bicarbonate dehydration activities of human CA I and CA II were also investigated in the four buffers mentioned above, at equivalent concentrations. No effect was demonstrated for CA II. CA I, however, responded to both phosphate and mops, as illustrated in Figure 4.3; 5.0 mM phosphate produced a 60% increase in activity, which did not alter appreciably up to 50.0 mM phosphate. Mops initiated no response at 5.0 mM, but increasing the concentration of mops produced an equivalent increase in activation.

Christiansen and Magid (1970) demonstrated a threefold increase in K_M and k_{cat} values for human CA I with phosphate (25 mM) and also confirmed that CA II does not respond to this anion. Furthermore, they confirmed the inert nature of hepes buffer in assays of CA I and CA II.

FIGURE 4.3 The Effects of Different Buffers on the Bicarbonate Dehydration Activity of Human CA I



Bicarbonate dehydration activity measurements were assayed in 30 mM HCO_3^- , pH 7.1, 2°C. (●-●) phosphate; (▲-▲) hepes; (□-□) pipes; (○-○) mops.

4.3 COMPARATIVE KINETIC STUDIES OF CARBONIC ANHYDRASE ISOENZYMES

In a previous comparative study in this laboratory, the K_M values towards bicarbonate for horse CA I, CA II and CA III were determined under 'optimal' conditions in a reaction mixture containing 13 mM phosphate and 5 mM EDTA (Hansen and Magid, 1965; Magid and Turbeck, 1968). These results are shown in Table 4.1, together with the relative activity measurements (k_{cat}/K_M) (Chegwidden *et al*, 1986). It was noted at the time that the relative activity of equine CA III as compared to horse CA I and CA II was surprisingly high when contrasted to data from other species. The CO_2 hydration activities and K_M s for three human isoenzymes are included in the table for comparison, although these values were determined using a stop flow apparatus and 14 mM barbitol buffer with no inclusion of phosphate (Sanyal *et al*, 1982). The horse kinetic parameters for CA I and CA II are comparable to those for human CA I and CA II (Magid, 1968).

In view of the activation by phosphate outlined in the previous section, it is possible that phosphate may have been a contributory factor in the high relative activity value obtained for horse CA III. The K_M value for human CA III towards bicarbonate using the same conditions as were employed for the equine isoenzymes ie 13 mM phosphate was too high to measure accurately by the pH stat method.

TABLE 4.1 Kinetic Parameters for the Bicarbonate Dehydration
Reaction of Horse and Human CA Isoenzymes

| | K_M mM | k_{cat} s ⁻¹ x 10 ³ | k_{cat}/K_M M ⁻¹ s ⁻¹ |
|---|----------|--|--|
| HCO ₃ ⁻ DEHYDRATION | | | |
| Horse CA I | 45 | 2.4 | 5.3 x 10 ⁴ |
| Horse CA II | 35 | 42.0 | 1.2 x 10 ⁶ |
| Horse CA III | 50 | 7.8 | 1.6 x 10 ⁵ |
| Human CA I* | 50 | 10.0 | 0.2 x 10 ⁶ |
| Human CA II | 30 | 110.0 | 3.7 x 10 ⁶ |
| CO ₂ HYDRATION** | | | |
| Human CA I | 4.4 | 29.0 | 6.6 x 10 ⁶ |
| Human CA II | 10.5 | 236.0 | 23.0 x 10 ⁶ |
| Human CA III | 45.0 | 8.9 | 0.2 x 10 ⁶ |

* Magid (1968)

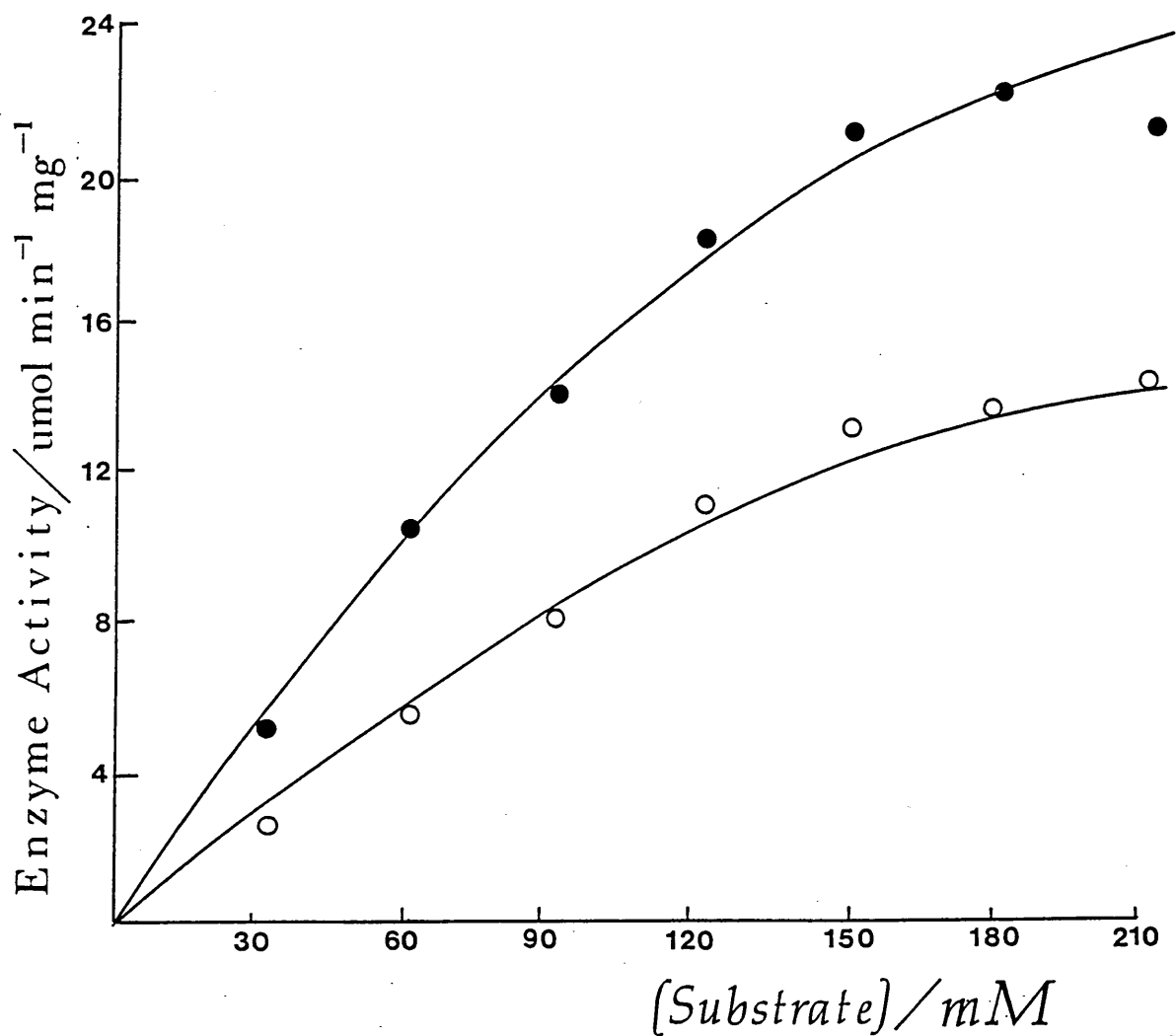
** Sanyal et al (1982)

In order to determine the effect of phosphate on the kinetic parameters of the muscle isoenzyme, these kinetic values were determined using either 10 mM hepes or 10 mM phosphate as the sole buffering system for chicken CA III. Whilst accurate and reproducible measurements were difficult to establish, it became apparent that phosphate probably decreases K_M , but has little effect on k_{cat} . A typical set of Michaelis-Menten plots are illustrated in Figure 4.4. The complementary kinetic parameters are listed in Table 4.2. The K_M in phosphate is approximately half that determined in hepes buffer, whereas k_{cat} remains essentially the same. Thus, the relative rate (k_{cat}/K_M) increases in 10 mM phosphate from 0.6×10^4 to $1.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$.

TABLE 4.2 Effect of P_i on K_M and k_{cat}/K_M of Chicken CA III

| | $K_M \text{ mM}$ | $k_{cat} \text{ s}^{-1}$ $\times 10^3$ | k_{cat}/K_M $\text{M}^{-1} \text{ s}^{-1}$ |
|-----------------|------------------|---|---|
| 10 mM Hepes | 333 | 1.9 | 0.6×10^4 |
| 10 mM Phosphate | 186 | 2.2 | 1.2×10^4 |

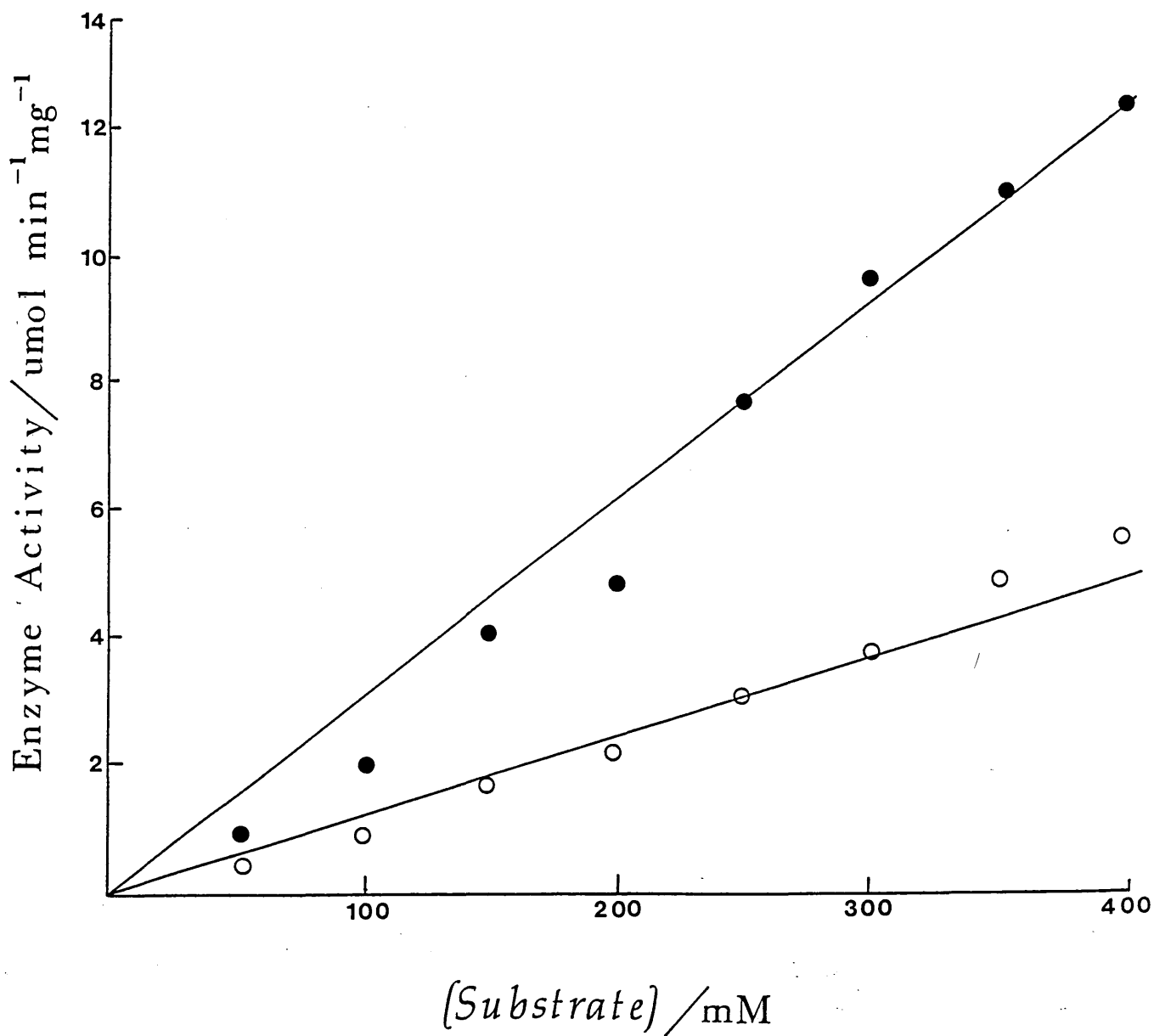
FIGURE 4.4 Michaelis-Menten Plot of the HCO_3^- Dehydration Reaction Catalysed by Chicken CA III



Enzyme activity was measured using 1 M H_2SO_4 (titrant) in either 10 mM phosphate buffer (●) or 10 mM hepes buffer (○). $[\text{E}] = 0.3 \mu\text{M}$.

A characteristic set of kinetic data for human CA III is also presented (Figure 4.5). The plots establish a linear relationship between activity and substrate concentration up to a [S] of 0.4 M. Although determination of K_M values are impractical it is clear from the graph that activity is considerably higher in phosphate than hepes.

FIGURE 4.5 Michaelis-Menten Plot of the HCO_3^- Dehydration Reaction Catalysed by Human CA III



Enzyme activity was measured using 1 M H_2SO_4 titrant (50-200 mM NaHCO_3) and 2 M H_2SO_4 titrant (250-400 mM NaHCO_3) in either 10 mM phosphate buffer (●) or 10 mM hepes buffer (○). $[\text{E}] = 0.1 \mu\text{M}$.

4.4 EFFECT OF ANIONS AND PHOSPHORYLATED METABOLITES ON HCO_3^- DEHYDRATION ACTIVITY OF MUSCLE ISOENZYMES

Consequent upon the finding that HCO_3^- dehydration activity of CA III is enhanced by the inclusion of phosphate buffer in the reaction medium, a number of other anions were examined for their effect on this reaction. In addition, a selection of intermediate metabolites of the glycolytic pathway of reactions, adenine nucleotides and other phosphorylated intermediates were also investigated. Initially these metabolites and ions were incorporated directly into the reaction chamber, but later some studies were conducted with prior incubation of metabolite/anion. All the measurements were performed at 30 mM [S]. Whilst 30 mM [S] was not an ideal [S] kinetically, and below the k_M values of human and chicken CA III, it did permit the determination of reliable, reproducible and defined measurements, an essential prerequisite for comparative analysis. At 30 mM [S] activity measurements were proportional to k_{cat}/k_M for human CA III.

4.4.1 Addition of Anions

N Polyvalent or monovalent ions with similar configurations spacially to the interacting phosphate ions were selected and included in the reaction medium together with 10 mM hepes. The results are listed for chicken and human CA III in Tables 4.3 and 4.4 respectively.

TABLE 4.3 Effect of Anions on the Bicarbonate Dehydration Activity of Chicken CA III

| | Conc mM | % Act | % Inh |
|---|---------|-------|-------|
| NaH ₂ PO ₄ /Na ₂ HPO ₄ buffer | 5.0 | 290 | - |
| K ₃ PO ₄ ·H ₂ O | 5.0 | 290 | - |
| SO ₄ ⁻⁻⁻ | 5.0 | - | 50 |
| CH ₃ COO ⁻ | 5.0 | - | 30 |
| HAsO ₄ ⁻⁻⁻ | 5.0 | - | - |
| B ₄ O ₇ ⁻⁻⁻ | 5.0 | - | - |
| NO ₃ ⁻ | 5.0 | - | - |
| HSO ₃ ⁻ | 1.0 | 163 | - |
| | 2.5 | 350 | - |
| HSO ₄ ⁻ | 5.0 | - | 20 |
| P ₂ O ₇ ⁻⁻⁻ | 1.0 | 140 | - |
| | 5.0 | 280 | - |
| | 10.0 | 307 | - |

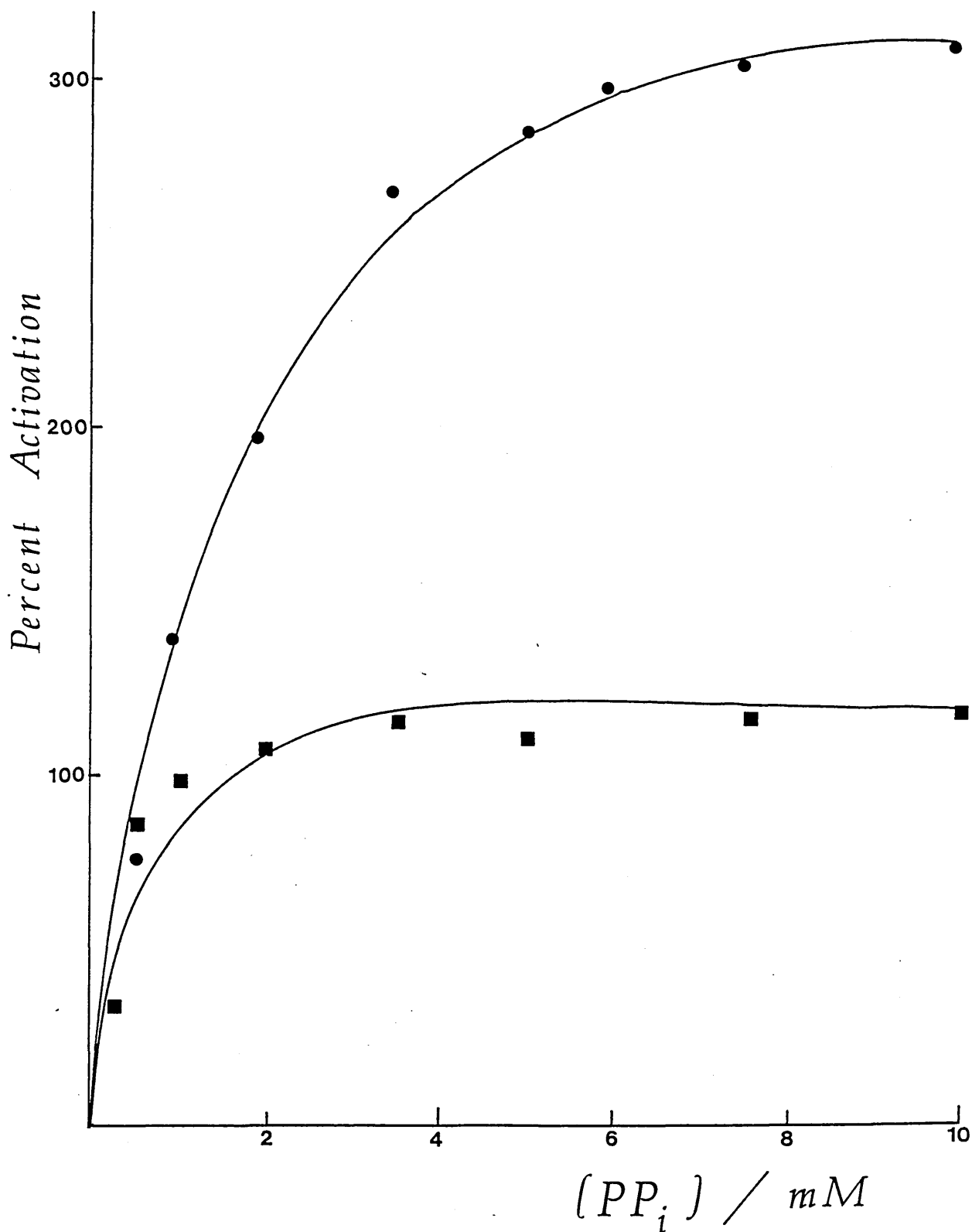
TABLE 4.4 Effect of Anions on the Bicarbonate Dehydration Activity of Human CA III

| | Conc mM | % Act | % Inh |
|---|---------|-------|-------|
| NaH ₂ PO ₄ /Na ₂ HPO ₄ buffer | 5.0 | 100 | - |
| P ₂ O ₇ ⁻⁻⁻ | 1.0 | 113 | - |
| | 5.0 | 107 | - |
| | 10.0 | 112 | - |
| HSO ₃ ⁻ | 1.0 | 167 | - |
| | 3.0 | 263 | - |
| HSO ₄ ⁻ | 5.0 | - | 46 |

Interestingly, hydrogen sulphite, at concentrations as low as 2.5 mM activated chicken CA III by as much as 350% and pyrophosphate by 140% at 1.0 mM. Arsenate, borate and nitrate were without effect, whereas sulphate, hydrogen sulphate and acetate were slightly inhibitory. A similar pattern prevailed for the human enzyme.

With the exclusion of hydrogen sulphite, the percentage increase for the human enzyme was much less than for the chicken protein. This difference in magnitude is illustrated by comparing the profile of activation by pyrophosphate for the two enzymes. Modification by this anion plateaus at a lower concentration in the human as compared to the chicken (Figure 4.6). Therefore, generally chicken CA III appeared to respond to a greater degree at 30 mM [S] than its human counterpart.

FIGURE 4.6 The Effects of Sodium Pyrophosphate on the HCO_3^- Dehydration Activity of Chicken and Human CA III.



Enzyme activity was measured using 0.1 M H_2SO_4 titrant, 30 mM NaHCO_3 and 10 mM hepes buffer. Assays were performed at pH 7.1, 2°C. [Chicken CA III] = 0.3 μM (●); [Human CA III] = 0.3 μM (■). k_a chicken CA III = 1.65 mM SE 0.182, k_a human CA III = 0.24 mM SE 0.066

4.4.2 Addition of Phosphorylated Esters

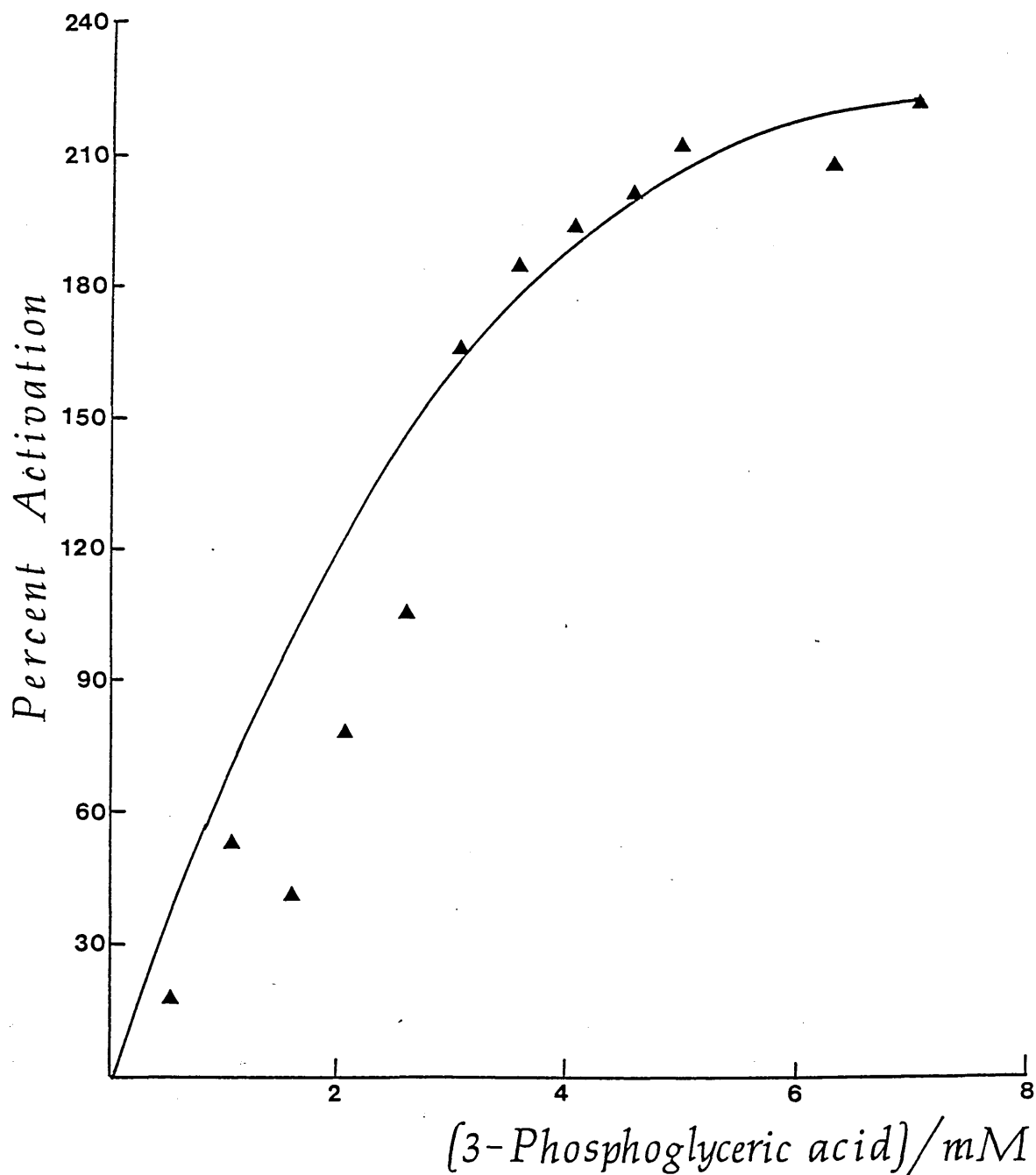
Three intermediates of the glycolytic pathway, namely 3-phosphoglycerate (3-PG), 1,3-bisphosphoglycerate (1,3-BPG) and phosphoenolpyruvate (PEP) were selected and their Tris or sodium salts introduced into the assay. It is clear from Table 4.5 that all three esters enhanced HCO_3^- activity of chicken CA III by 200-300%. 1,3-BPG was a particularly potent activator, conferring a 3-4 fold activation at 3 mM. The concentration at which optimal percentage activation occurred was determined by constructing a concentration response curve. An example (3-PG) is included for reference (Figure 4.7).

Several phosphorylated metabolites that play an integral part in muscle metabolism were also investigated, these included phosphocreatine, pyridoxal phosphate and inosine 5'-monophosphate. None of these intermediates effected bicarbonate dehydration activity of chicken CA III.

TABLE 4.5 Effect of Phosphorylated Esters on the Bicarbonate Dehydration Activity of Chicken CA III

| | Concentration mM | % Activation | Concentration in Resting Muscle mM |
|--------------------------------------|---------------------|--------------|---------------------------------------|
| 3-Phosphoglycerate (3-PG) | 2.0 | 86 | - |
| | 4.0 | 190 | - |
| | 7.5 | 210 | - |
| | 0.38 | 115 | - |
| 1,3-Bisphosphoglycerate (1,3-BPG) | 1.13 | 269 | - |
| | 2.26 | 338 | - |
| | 3.01 | 369 | - |
| Phosphoenolpyruvate (PEP) | 0.86 | 95 | - |
| | 1.72 | 150 | - |
| | 2.15 | 186 | - |
| | 3.01 | 232 | - |
| Phosphocreatine | 5.0 | - | 25 |
| Inosine 5'-monophosphate | 6.7 | - | - |
| Pyridoxal phosphate | 6.7 | - | - |

FIGURE 4.7 Concentration Response Curve of Chicken CA III
(HCO_3^- Dehydration) to D(-)3-phosphoglyceric Acid (3-PG)



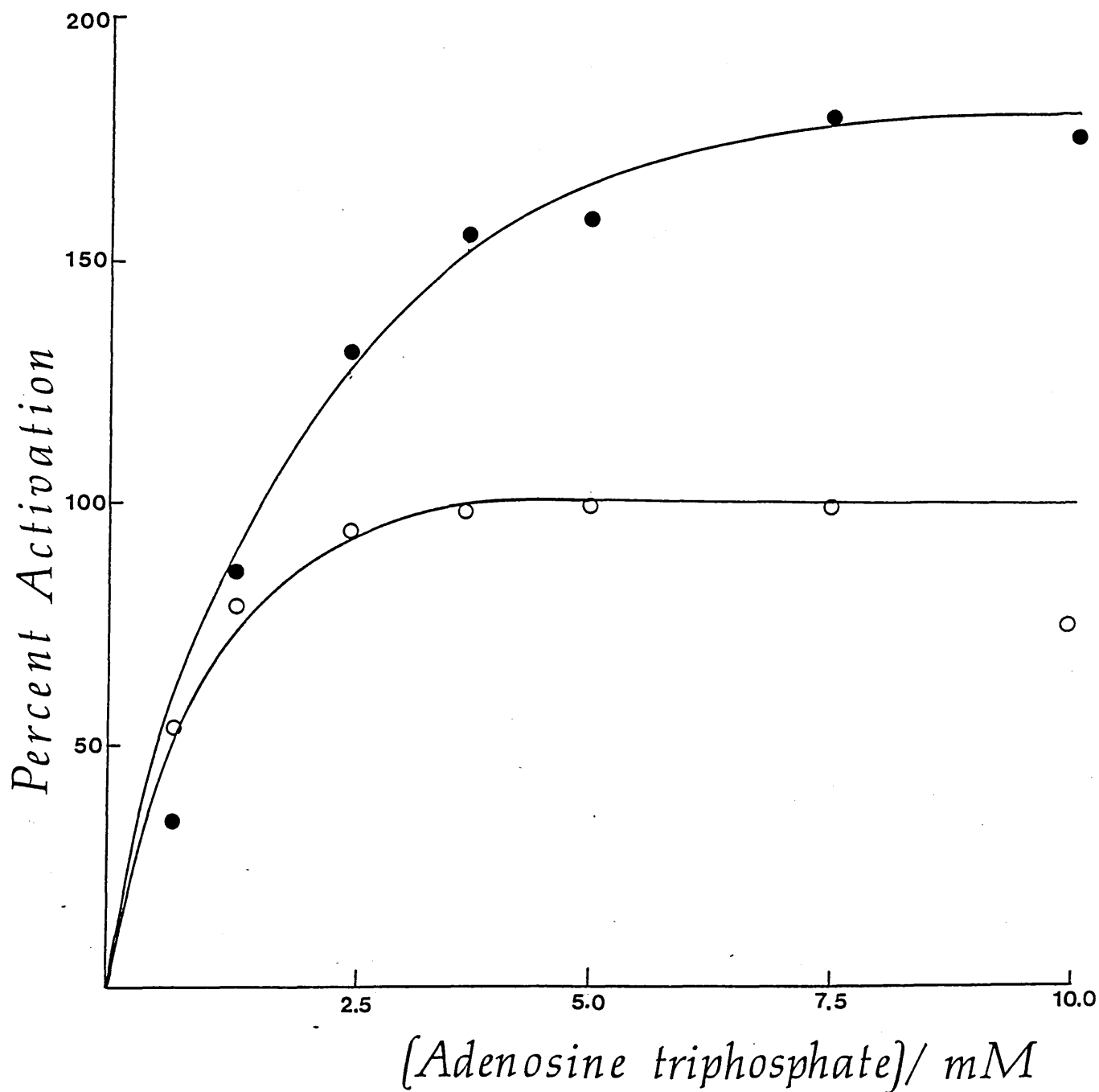
Enzyme activity was measured using 0.1 M H_2SO_4 titrant, 30 mM NaHCO_3 and 10 mM hepes buffer. Assays were performed at pH 7.1, 2°C.

The adenine nucleotides, ATP, ADP, AMP and cAMP were examined for their effect on the HCO_3^- dehydration activity of human and chicken CA III, and the results can be seen in Table 4.6. ATP and ADP enhanced activity in both isoenzymes, whereas AMP and cAMP were without effect. A profile showing activation by ATP in the two isozymes is included (Figure 4.8) and again the avian enzyme exhibits a markedly higher response.

TABLE 4.6 Effect of Adenine Nucleotides on the HCO_3^- Dehydration Activity of Human and Chicken CA III

| Percentage Activation | | | | |
|-----------------------|---------|-----------------|-------------------|--------------------------------------|
| | Conc mM | Human CA III | Chicken CA III | Cellular Conc mM (resting muscle) |
| ATP | 2.5 | 94 | 130 | 4 |
| | 7.5 | 100 | 181 | |
| ADP | 5.0 | 59 | 102 | 0.013 |
| AMP | 5.0 | - | - | |
| cAMP | 5.0 | - | - | |

FIGURE 4.8 Concentration Response Curve of the HCO_3^- Dehydration Activity of Chicken and Human CA III to Adenosine Triphosphate (ATP).



pH-stat assays were performed in 10 mM hepes buffer using 30 mM NaHCO_3 at pH 7.1, 2°C. Chicken CA III (●); Human CA III (○).

a) Inorganic Phosphate Studies

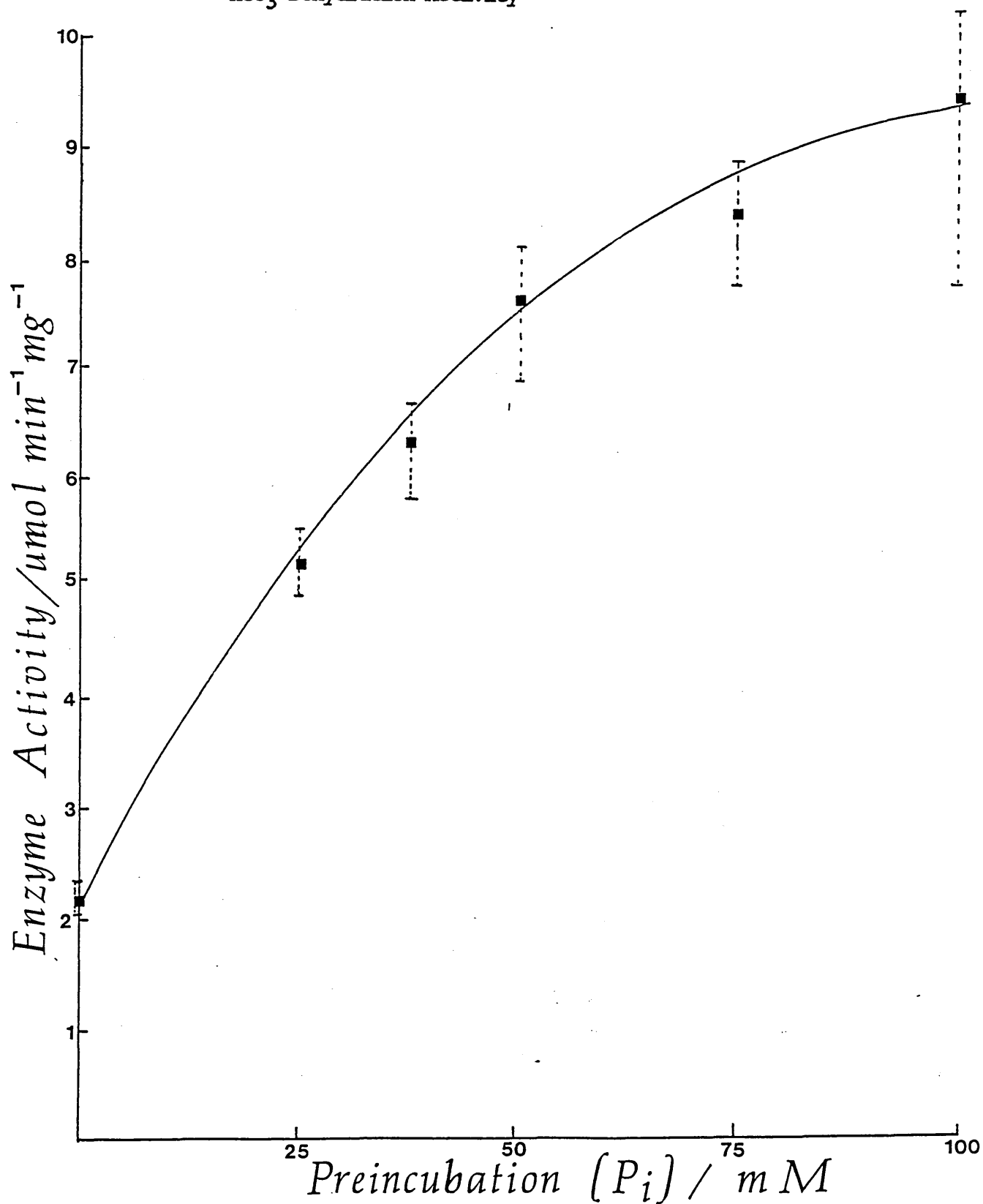
The possibility of ATP, ADP, 3-PG, 1,3-BPG and PEP hydrolysis liberating free phosphate either before or during pH-stat assays existed, so a number of inorganic phosphate determinations were conducted. The inorganic content of the phosphoryl esters was found to be negligible, less than 0.013% for ATP, 0.07% for ADP and none was detected for the glycolytic pathway intermediates. The nannogram quantities detected in ATP and ADP would be unlikely to account for the considerable activation of HCO_3^- dehydration demonstrated with these metabolites.

No phosphate was released during the assays, even after complete depletion of substrate. Furthermore, in the absence of substrate, chicken and human CA III did not exhibit any phosphatase activity with any of the five metabolites mentioned above.

4.4.3 Preincubation of Chicken CA III with Phosphate

Figure 4.9 shows the effect of preincubation of chicken CA III with a 5-50 mM concentration range of phosphate contained in 50 mM borate buffer pH 8.3, for a period of 160 minutes. The final P_i concentration in the HCO_3^- dehydration assay was 0.25-1.00 mM. Bicarbonate dehydration activity was enhanced at phosphate concentrations that have previously yielded a negative result when added to the reaction medium (section 4.2).

FIGURE 4.9 The Effect of Preincubating CA III in Phosphate on the HCO_3^- Dehydration Activity



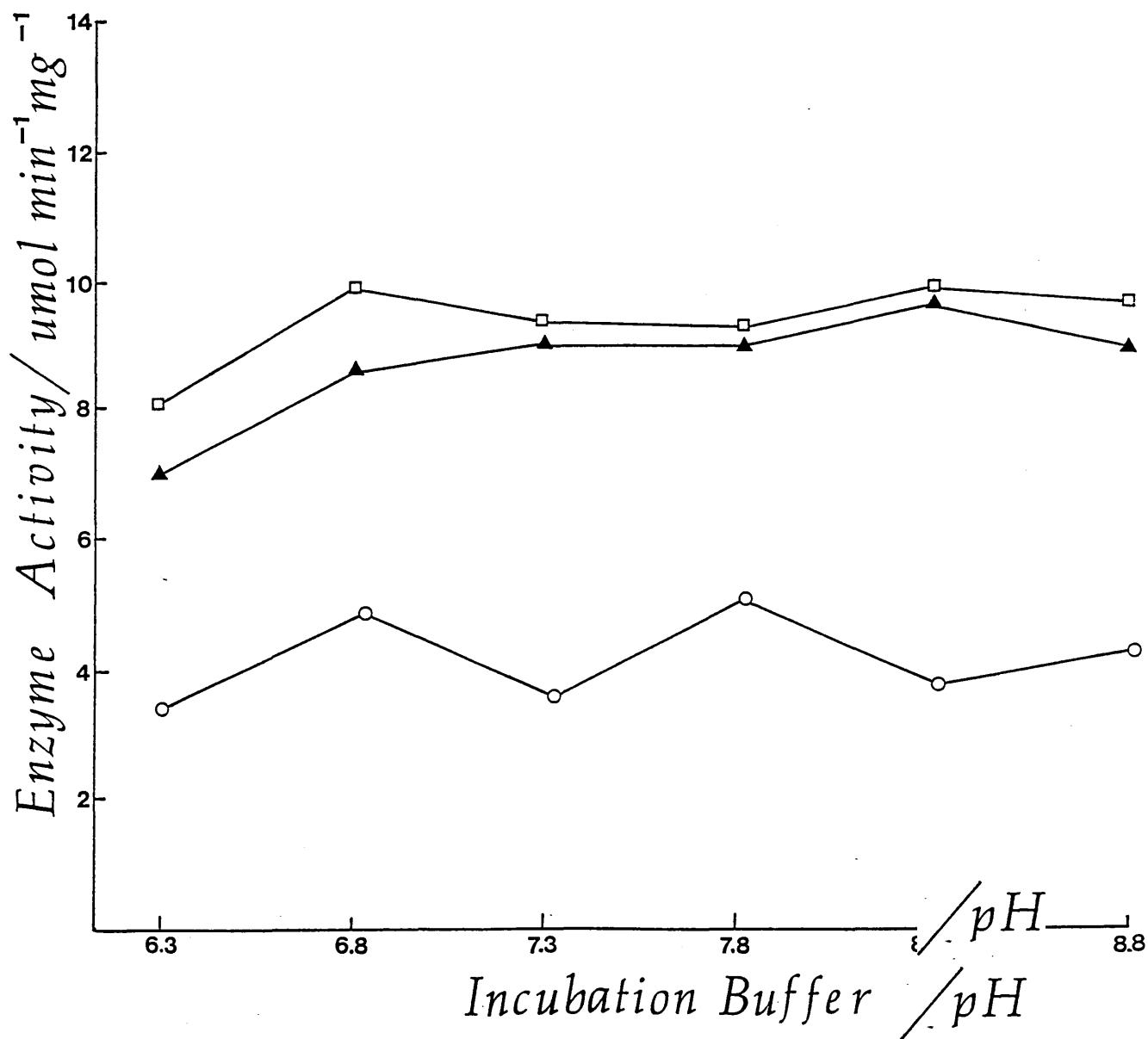
Chicken CA III was preincubated in 25-100 mM P_i contained in 50 mM borate buffer pH 8.3 for 160 min at 25°C. 0.1 aliquots were assayed as described in section 2.8.1. Final $[\text{P}_i]$ in assay 0.25-1.0 mM. The bars show the maximum/minimum activities from four experiments.

a) Effect of Buffer System and pH

The possibility that the activation resulting from preincubation of enzyme with phosphate could be due to the borate buffer or the high pH was precluded (Figure 4.10).

Irrespective of the pH of the incubation buffer system between 6.3 and 8.8 or the buffer itself (Tris or borate), preincubation of chicken CA III with 100 mM phosphate resulted in enhanced HCO_3^- activity. All the assays were conducted at pH 7.1. Adding the equivalent phosphate concentration (1.00 mM) directly to the assay produced a basal control activity measurement.

FIGURE 4.10 The Effect of Preincubating Chicken CA III in Buffers of Varying pH Values on the HCO_3^- Dehydration Activity



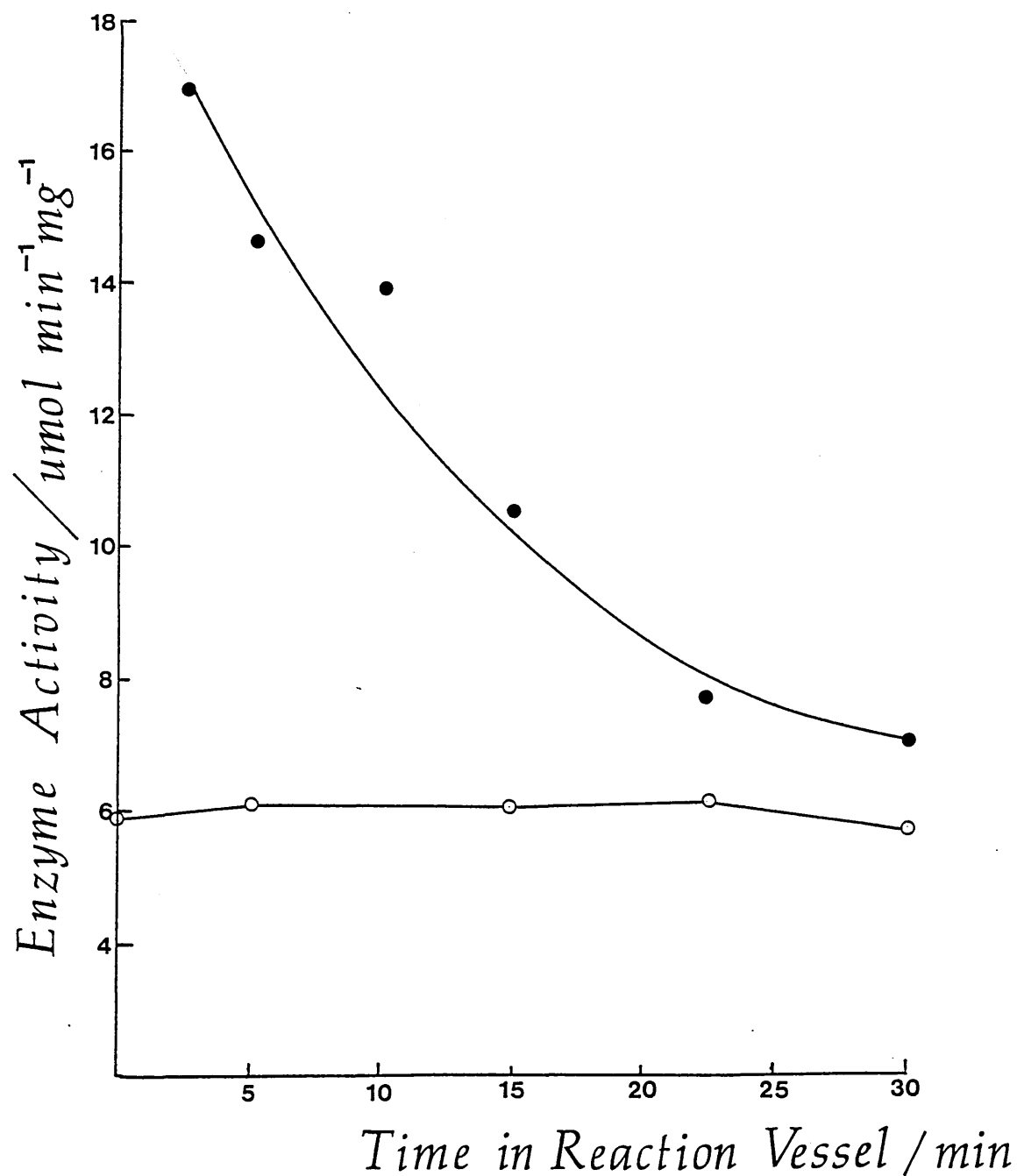
Chicken CA III was preincubated in 5 mM Tris (○), 100 mM phosphate in 50 mM borate (▲) or 100 mM phosphate in 5 mM Tris buffer (□) adjusted to pH 6.3-8.8 for 120 min at 25°C. 0.1 ml aliquots were assayed as described in section 2.8.1 at pH 7.1.

b) Dissociation of the Enzyme-Phosphate Complex

Chicken CA III was preincubated in 100 mM NaH_2PO_4 in Tris buffer as described. To determine the stability of this ionic interaction between enzyme and phosphate, the enzyme was transferred after incubation and left to bubble in the reaction vessel for various periods of time prior to assaying. In this instance, the substrate was added to initiate the velocity measurements rather than enzyme. Control samples in Tris buffer were afforded the same treatment.

Figure 4.11 illustrates the gradual dissociation of the enzyme-phosphate complex and the concomitant reduction in activity. Equilibration is apparently not immediate. A three fold activation is still evident after two minutes rigorous stirring, and even after fifteen minutes treatment, some phosphate must still be bound to a proportion of the CA III molecules. The basal level attained after 30 minutes agitation clearly confirms the previous finding, that 1.0 mM phosphate has no significant effect on the activity of chicken CA III when equilibrated in the assay mixture.

FIGURE 4.11 Time Course Curve of the HCO_3^- Dehydration Activity of Chicken CA III Preincubated in Phosphate



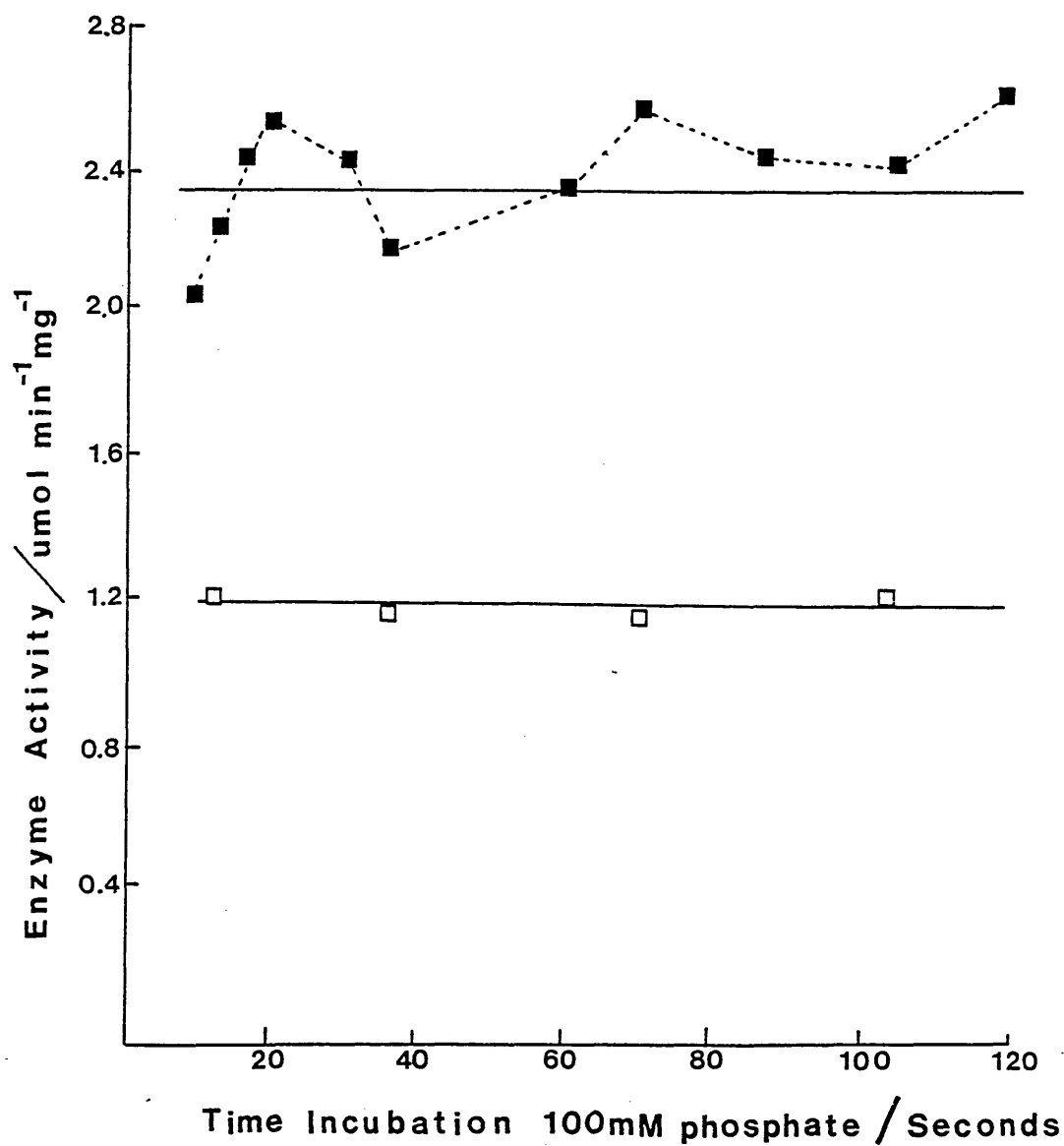
Enzyme was preincubated in 100 mM P_i contained in 5 mM Tris buffer pH 7.1 for 2 h at 25°C (●). Control sample was left in 5 mM Tris buffer (○). 0.1 ml enzyme samples were transferred to the pH-stat assay vessel (10 ml volume) and left for 2—30 min at 2°C prior to determining the HCO_3^- dehydration activity (section 2.8.1).

c) Formation of the Enzyme-Phosphate Complex

It appears that complete dissociation of the enzyme-phosphate complex requires at least 30 minutes, presumably for conformational changes to occur. An experiment was designed to ascertain whether formation of the complex required an equivalent period of time. Chicken CA III was added to the phosphate incubation mixture, mixed, then immediately withdrawn and assayed. The time taken to accomplish this procedure was precisely timed and recorded.

The results (Figure 4.12) imply that the formation of the enzyme-phosphate complex, as deduced from enhanced activity, occurs rapidly within twelve seconds and no appreciable increase in activity ensues over a two minute period. Thus, the formation of the enzyme-phosphate complex is instantaneous, whilst the dissociation occurs more slowly.

FIGURE 4.12 The Effect of Preincubation Time in Phosphate on the Bicarbonate Dehydration Activity of Chicken CA III



Chicken CA III was mixed with either 100 mM phosphate in 5 mM Tris 7.1 (■) or 5 mM Tris pH 7.1 (□), then 0.1 ml was immediately assayed for HCO₃⁻ dehydration activity in 10 mM hepes pH 7.1 as described (2.8.1).

4.5 pH-RATE PROFILES FOR THE BICARBONATE DEHYDRATION REACTION IN HEPES AND PHOSPHATE BUFFERS

Whilst maintaining strict pH at 7.1, the ionic species of a $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer should be accurately controlled. It was however, pertinent to discover the effect of altering the pH and thus the proportion of the ionic species PO_4^{3-} , HPO_4^{2-} , H_2PO_4^- and H_3PO_4 over a physiologically relevant range. So, activities of human and chicken CA III were compared in 10 mM hepes and 10 mM phosphate buffers at various pHs between 6.7 and 7.5. In addition, chicken CA III preincubated in 100 mM phosphate pH 7.1 was assayed over the same range.

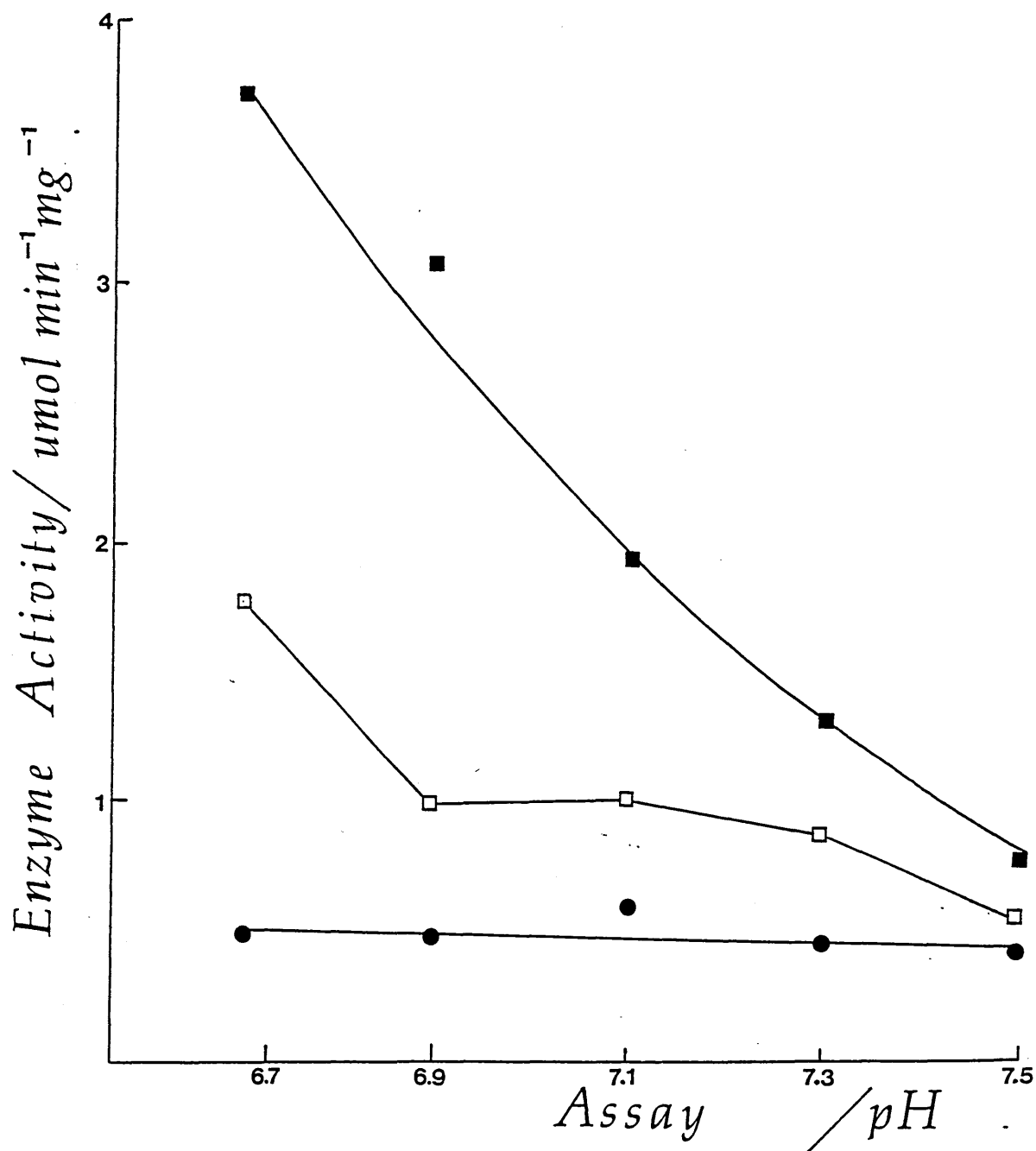
The results for chicken CA III in Figure 4.13 indicate that there is a marked increase in activation by phosphate with decreasing pH. Activity in hepes remains essentially constant over this pH range. The combined results of three experiments (Figure 4.14), suggest that at pH 6.7, 10 mM phosphate increases activity by as much as 600%.

Previous data (section 4.4.3 a) indicated that preincubating chicken CA III with 100 mM phosphate ranging in pH from 6.3 to 8.8 had little effect on the degree of activation, when assayed at pH 7.1 Figure 4.9. However, it appears from Figures 4.13 and 4.14, that preincubating chicken CA III with 100 mM phosphate pH 7.1 then assaying at different pHs, results in increased activation at lower pH values. A 200% increase was evident at pH 6.7. A more marked effect of pH was monitored in phosphate buffer. Thus muscle enzyme in contact with transient high levels of P_i at pH 7.0, could respond to a decrease in pH during exercise, even if the P_i level subsequently diminished.

Ren and his co-workers (1988) demonstrated that in bovine CA III, both k_{cat} and K_M were raised slightly with increasing pH, but k_{cat}/K_M varied little with pH. The buffers in this study were 1,2-dimethylimidazole, mops and 1-methylimidazole. Engberg et al, (1985) working on the same isoenzyme reported that activity was nearly independent of pH between 6.0 and 8.6.

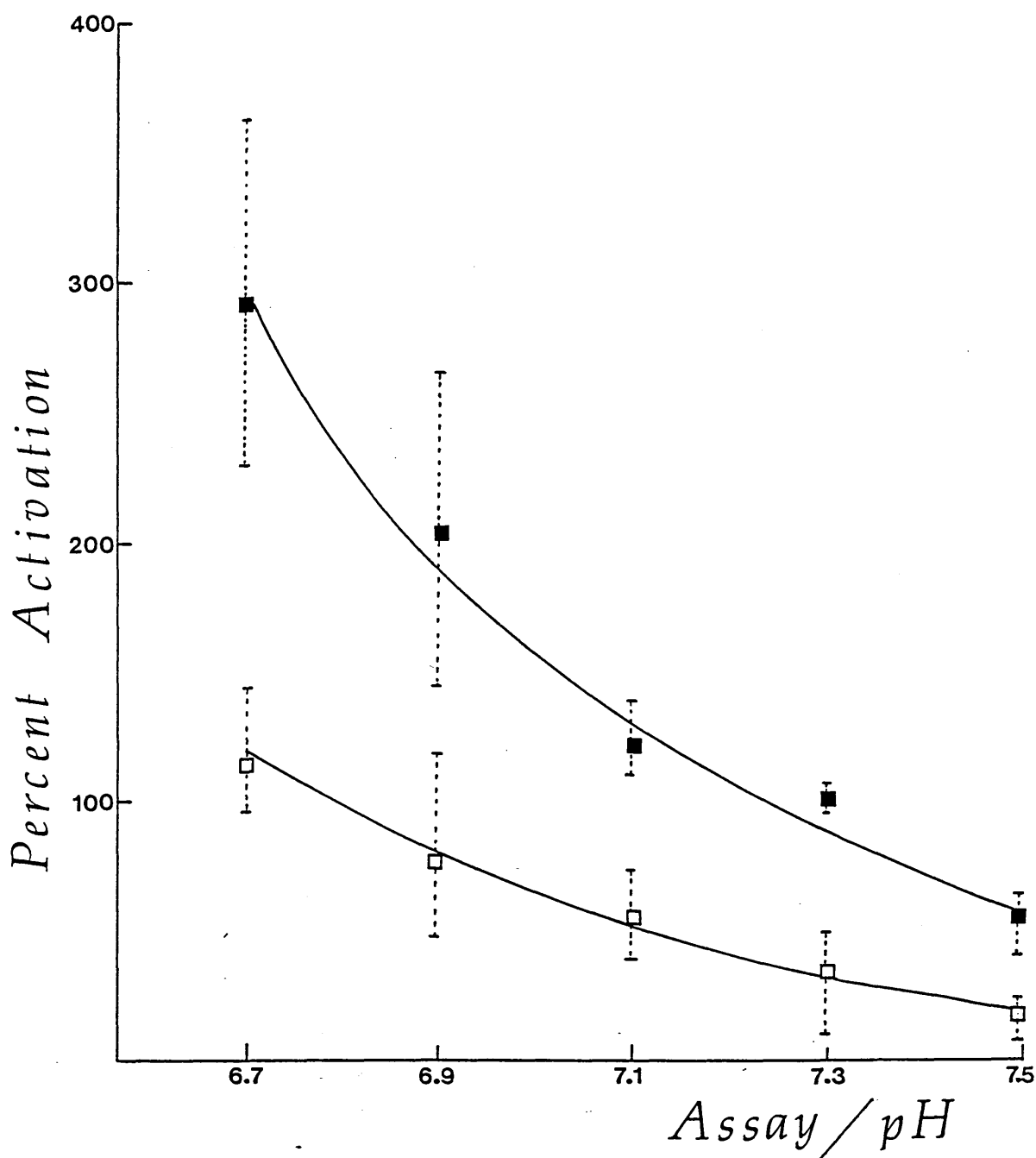
The pH-rate profile for human CA III illustrated in Figure 4.15 showed a different response to the chicken muscle isoenzyme. A decrease in activity was evident in both buffers. Thus, the percentage activation by phosphate remained essentially constant from pH 6.7-7.4. The decrease in activity with increasing pH was also mirrored in similar studies using pipes and mops buffers in this protein.

FIGURE 4.13 pH-Profile of the Bicarbonate Dehydration Activity of Chicken CA III Preincubated in Phosphate



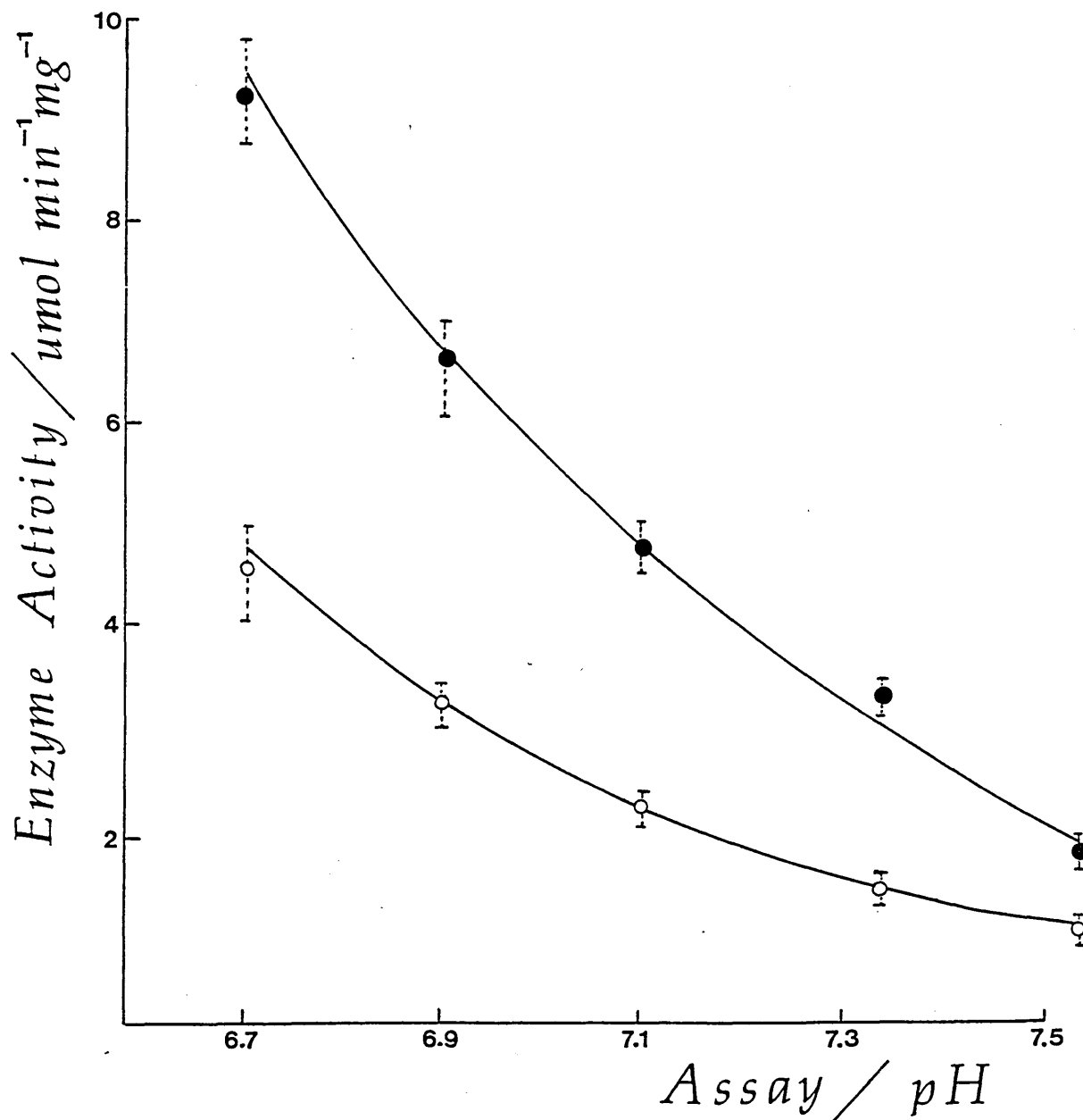
Enzyme was incubated in 100 mM phosphate in 5 mM Tris buffer (□) for 2 h at 25°C. 0.1 ml was assayed at pH 6.7–7.5 in 10 mM hepes as described in 2.8.1 (10 ml reaction volume, $[P_i] = 1 \text{ mM}$). Control samples in 5 mM Tris buffer were assayed in either 10 mM phosphate buffer (■) or 10 mM hepes buffer (●) (pH 6.7–7.5).

FIGURE 4.14 Comparison of the Effect of pH on the Activation of the HCO_3^- Dehydration Activity of Chicken CA III by Phosphate and by Preincubation in Phosphate



Enzyme was incubated in 100 mM phosphate in 5 mM Tris buffer pH 7.1 for 2 h at 25°C (□) and assayed at pH 6.7-7.5 in 10 mM hepes, $[\text{P}_i] = 1$ mM. Enzyme in 5 mM Tris (■) was assayed in 10 mM phosphate (pH 6.7-7.5). % Activation was calculated by comparison with measurements of control enzyme in hepes. The bars represent the maximum/minimum activation from three experiments.

FIGURE 4.15 Effect of pH on the Enhancement of HCO_3^- Dehydration Activity by Phosphate in Human CA III



Human CA III was assayed at pH 6.7–7.6 in 10 mM phosphate (●) or 10 mM hepes (○) as described in 2.8.1. Error bars $n = 5$.

4.6 INTERACTION OF PHOSPHATE WITH PHOSPHORYL ESTERS, MODIFIED ENZYME AND INHIBITORS (CHICKEN CA III)

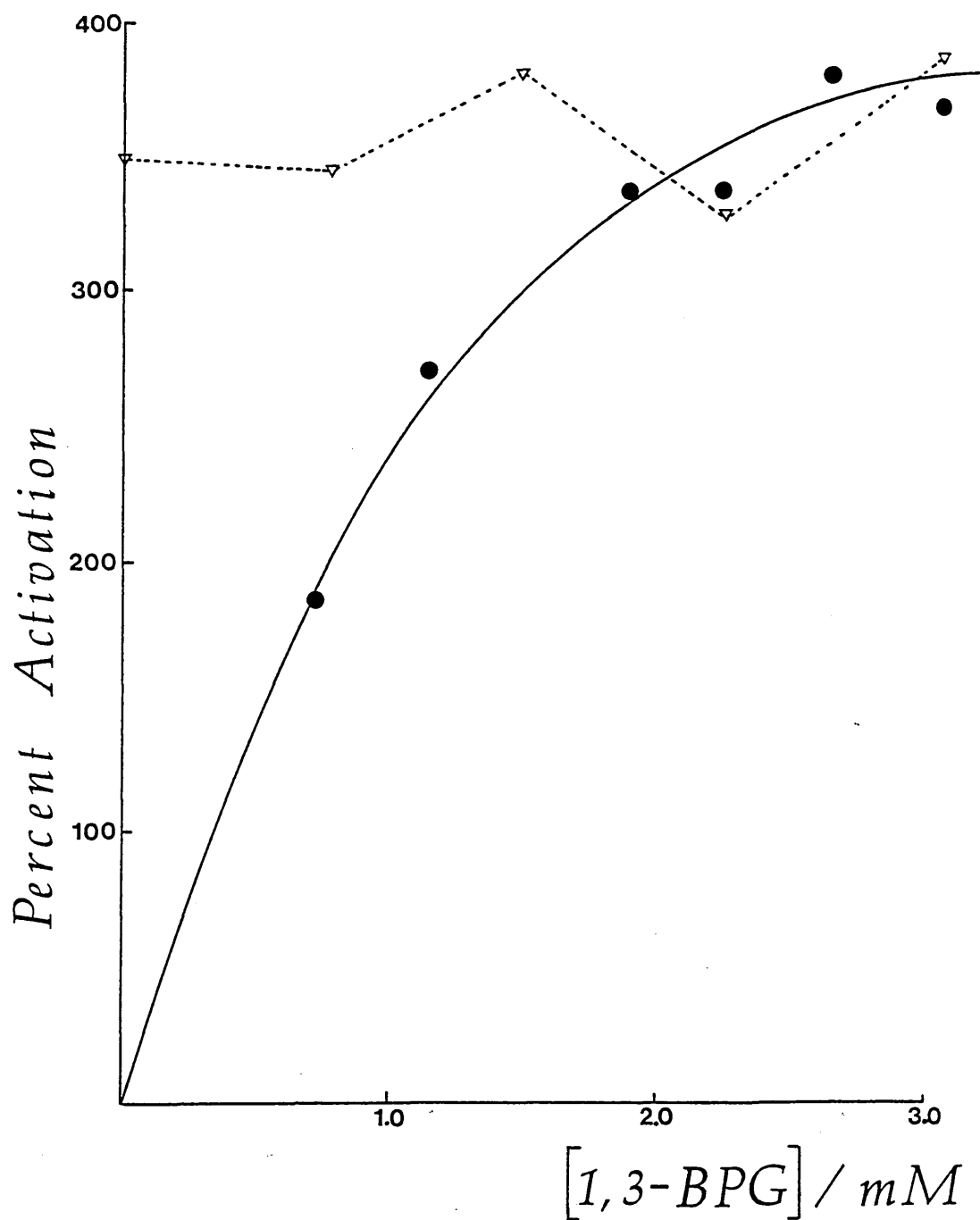
The nature of the phosphate-enzyme complex was investigated by analysing the effect of phosphate on previously modified enzyme or when an activator or inhibitor was included, in an attempt to discover whether these enzyme interactions were mutually exclusive or additive.

4.6.1 Interaction of Phosphate with Phosphoryl Esters

Phosphate concentration was maintained at 10 mM, while ATP, 3-PG, 1,3-BPG and PEP were increased in concentration. The molarity of esters correlated with the range producing enhancement of HCO_3^- dehydration activity (section 4.4.2). Each ester yielded similar results and a representative graph for 1,3-BPG is shown (Figure 4.16). The control curve with ester in hepes buffer displays a standard increase in activation with increasing metabolite. By contrast, an increase in ester when phosphate is present has no additional effect on activation.

It appears that once activated by 10 mM phosphate, chicken CA III cannot be activated further by these phosphorylated metabolites. This suggests that these esters are acting on the same part of the active site, perhaps by a common mechanism. Moreover, it indicates that 10 mM represents a concentration of maximal activation.

FIGURE 4.16 The Effect of Phosphate on the Activation of the HCO_3^- Dehydration Activity of Chicken CA III by 1,3-BPG.



The enzyme assay was performed as described (2.8.1) in 10 mM hepes buffer (●) or 10 mM phosphate buffer (▽) with increasing 1,3-biphosphoglycerate.

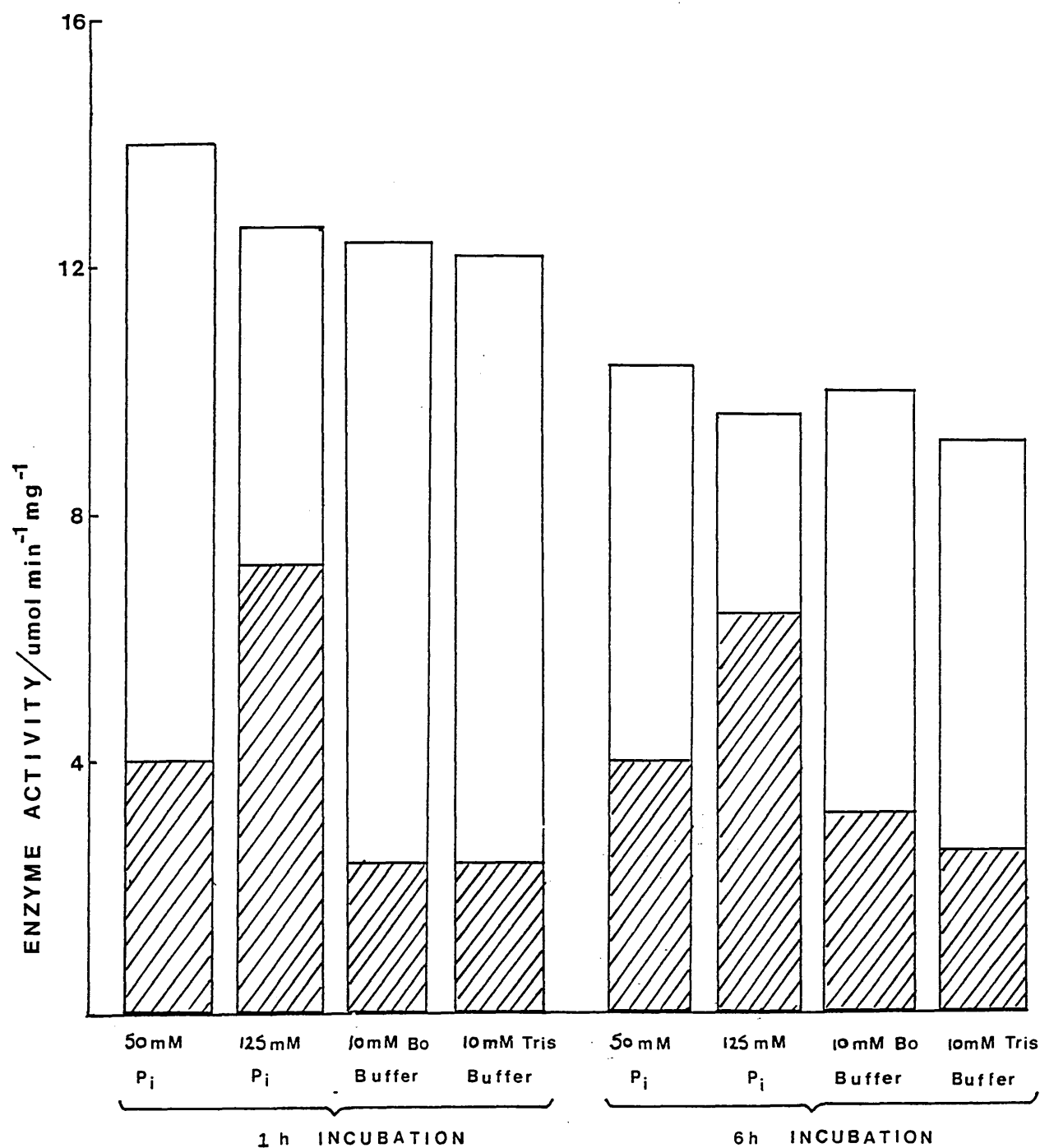
4.6.2 Interaction of Phosphate and Phosphate-Enzyme Complex



Chicken CA III was preincubated in 50 mM and 125 mM phosphate for one or six hours, then assayed in either hepes or phosphate buffer. The enzyme aliquots were diluted 1:100, representing a final phosphate concentration in the hepes assay of 0.5 mM and 1.25 mM respectively. The results are represented in histogram form in Figure 4.17.

Several conclusions can be made from this set of data. Firstly, incubating phosphate with enzyme increases HCO_3^- dehydration activity after one and six hours. The response is more pronounced in 125 mM phosphate. Secondly, addition of phosphate to enzyme already complexed to phosphate results in further activation. Finally, the maximal degree of activation produced by assaying in phosphate is similar in all the treatments irrespective of whether enzyme has bound phosphate. It is also worth noting that the propensity for responding to added phosphate is partially lost with time, since there is a general activity loss after six hours in phosphate whilst the measurements in hepes are constant.

The results from the previous section suggest that phosphate and ester activation might have a common mechanism, whereas this data indicates that prior treatment with phosphate does not preclude additional activation by this anion. Perhaps not all the enzyme molecules are modified, or there is a partial equilibrium upon transfer to the reaction vessel (complete dissociation is unlikely from previous results) or maybe there are two modes/sites of phosphate interaction.

FIGURE 4.17 Effects of Phosphate on the Bicarbonate Dehydration Activity of Chicken CA III by Preincubation and/or Inclusion in the Assay Mixture



Chicken CA III was assayed in  10 mM hapes buffer or  10 mM phosphate buffer.

4.6.3 Butanedione Modification

The unique positioning of arginine or lysine residues in the active site of the type III isoenzymes has been emphasised and the possibility that phosphorylated intermediates or ions might influence the regulation or control of this muscle protein in vivo is highly probable. Previous studies in this laboratory have shown that 2,3-butanedione, under strict conditions, specifically modifies arginine residues in the active site of chicken CA III. This BD-modification was found to enhance the esterase activity, but not the HCO_3^- dehydration activity (Tashian et al, 1984; Chegwidan et al, 1988). However, in these studies the pH-stat assays were performed in 13 mM phosphate, which in view of recent findings is likely to complicate the interpretation of the results.

a) Comparison of BD-Modified and P_i -Modified Chicken CA III

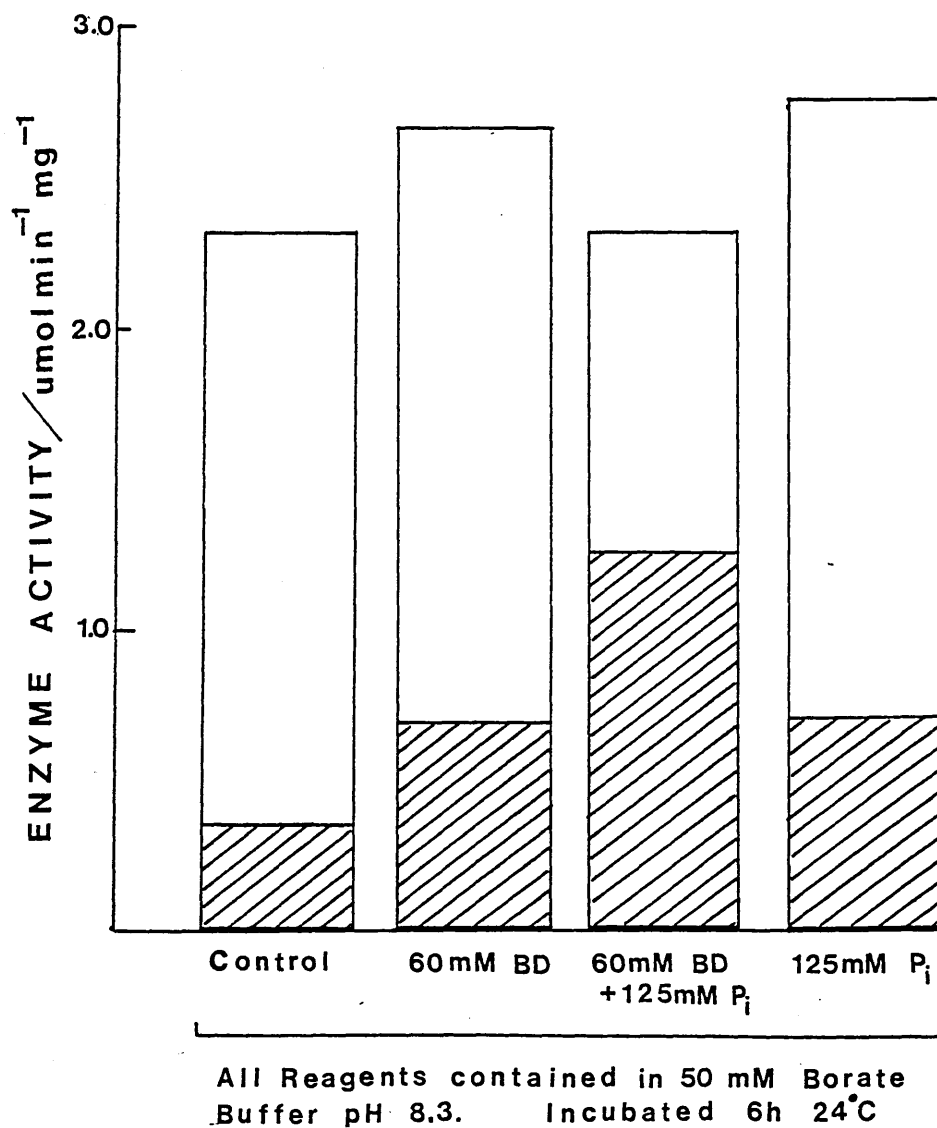
The HCO_3^- dehydration activity of chicken CA III modified by 60 mM butanedione was compared to enzyme modified by preincubation with 125 mM NaH_2PO_4 over a six hour period. Both modifiers were contained in 50 mM borate buffer pH 8.3 and left at room temperature. An additional sample included butanedione and phosphate. All the assays were performed in hepes and phosphate buffer.

The histogram shown in Figure 4.18 after six hours treatment indicates that both modifiers produce a similar degree of activation when assayed in hepes, and that both BD-modified and P_i -modified enzyme can be further activated by the addition of phosphate. It would appear from studying these two treatments that the mode of action might be similar eg a mechanism involving active site arginine

residues. Yet, when both modifying agents are acting simultaneously there is an increased effect. The time course plot (Figure 4.19) reveals an increased response in this sample over the first hour, which could be accounted for by a slow conformational change indicative of BD action. Notwithstanding the mode of action of BD, the theory that these modifiers act on the same site is not excluded, but there is an apparent additional complementary effect. It is tempting to speculate that BD-modification could unfold the polypeptide chain thus facilitating phosphate interaction at a site normally buried, or there are quite simply two response sites.

An extended study over a twenty four hour period demonstrated an essentially constant level of comparative activity from 6-24 h.

FIGURE 4.18 Effects of BD-modification and P_i -modification of Chicken CA III on the HCO_3^- Dehydration Activity.





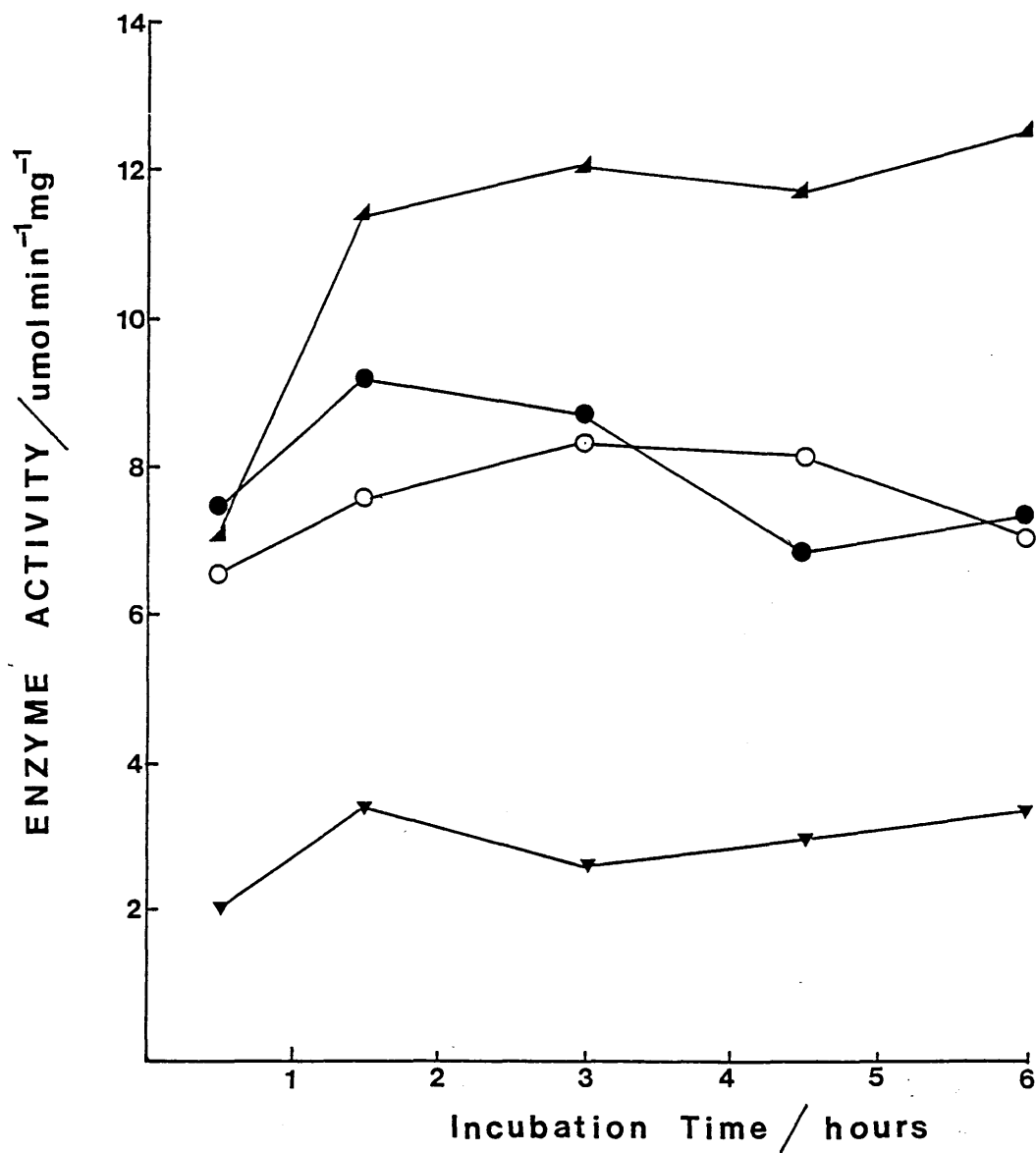
Chicken CA III was preincubated with either BD or P_i (as indicated) before assaying in  10 mM hepes buffer, or  10 mM phosphate buffer.

FIGURE 4.19 Simultaneous Effect of BD-modification and P_i -modification of Chicken CA III on the HCO_3^- Dehydration Activity



Enzyme treatments:- (○) 60 mM BD in 50 mM borate buffer; (●) 125 mM P_i in 50 mM borate buffer; (▲) 60 mM BD + 125 mM P_i in 50 mM borate buffer; (▼) control 50 mM borate buffer pH 8.3. All assays were performed in 10 mM hepes at 2°C, pH 7.1.

b) Effect of Phosphate on BD-Modified Chicken CA III

Phosphate (125 mM) was added to fully BD-modified chicken CA III, and assayed at 40 min intervals in 10 mM hepes buffer. The results in Table 4.7 show that after 40 min there is essentially no difference in activity between the BD-modified sample and the BD-modified sample with added phosphate. A slightly lower activity is demonstrated in the BD-modified enzyme with P_i after 160 min. Therefore pretreatment of enzyme with BD excludes further activation by phosphate modification, whereas, when phosphate and BD are in competition a complex interaction exists. Thus far, it appears that phosphate binds to the same sites as BD ie arginine residues, with concomitant enhancement of HCO_3^- dehydration activity, but phosphate has an additional site or effect when added to the assay medium.

It is worth noting that in the absence of P_i , BD-modification enhances activity by approximately 100%. In previous studies containing phosphate buffer, it was concluded that P_i enhanced the esterase activity towards 4-NPA but not the HCO_3^- dehydration activity.

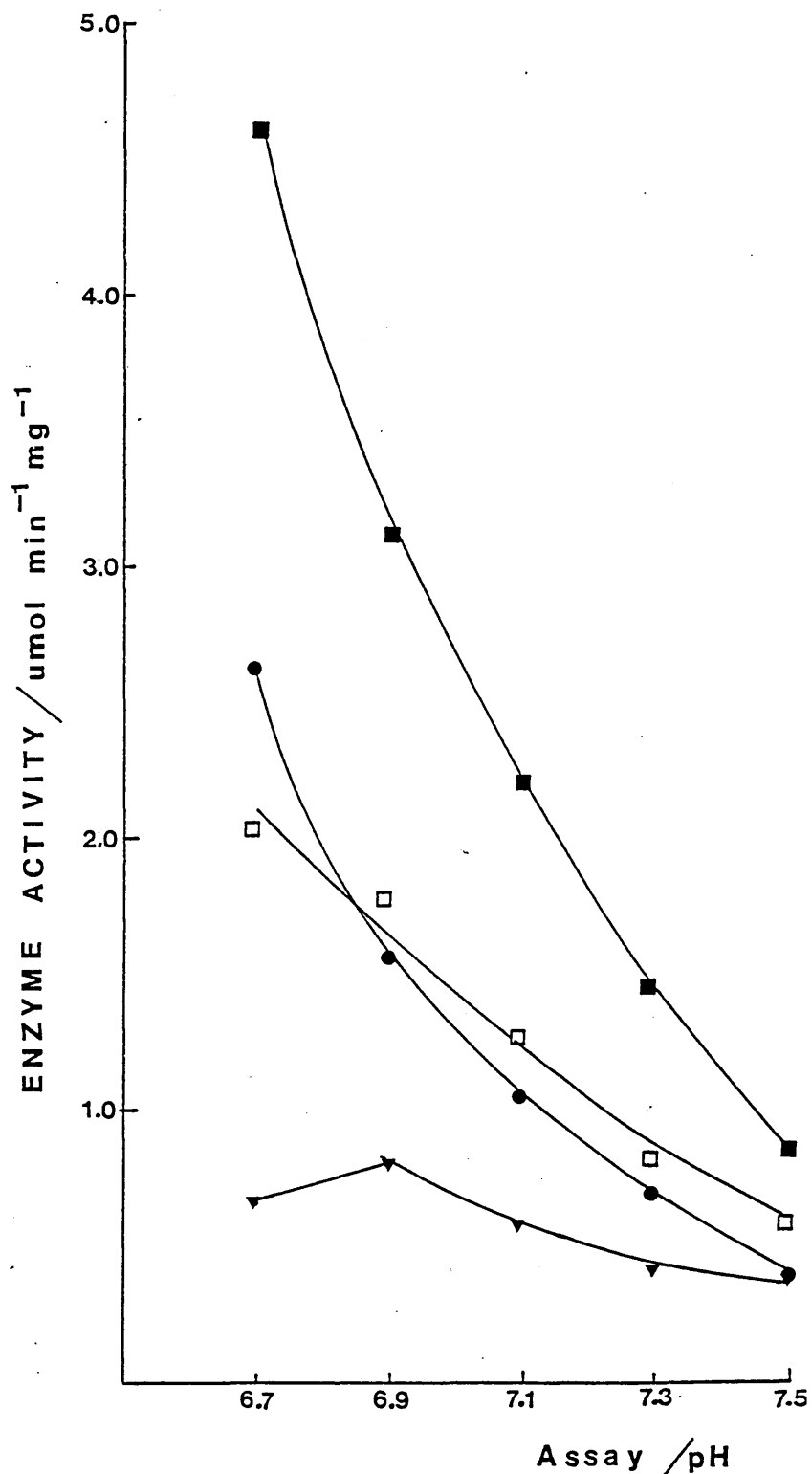
TABLE 4.7 Effect of P_i on the HCO_3^- Dehydration Activity of BD-modified Chicken CA III

| | Activity $\text{mmol min}^{-1} \text{mg}^{-1}$ | | |
|----------------------------|--|---------|---------|
| | <u>Time after addition P_i</u> | | |
| | 40 min | 160 min | 220 min |
| BD-modified | 7.5 | 7.8 | 8.3 |
| BD-modified + 125 mM P_i | 7.3 | 6.3 | 7.2 |
| Control borate buffer | 3.8 | 3.8 | 3.9 |

c) Comparison of the pH-profiles of P-modified Enzyme and BD-modified Enzyme

The degree of activation resulting from preincubation of chicken CA III with 125 mM phosphate pH 7.1 and assaying at pH values between 6.7 and 7.5, is essentially the same as modifying chicken CA III with BD (Figure 4.20). Both treatments produce a concomitant increase in activity with decreasing pH.

FIGURE 4.20 Effect of pH on the HCO_3^- Dehydration Activity of Chicken CA III after Preincubation with P_i and BD



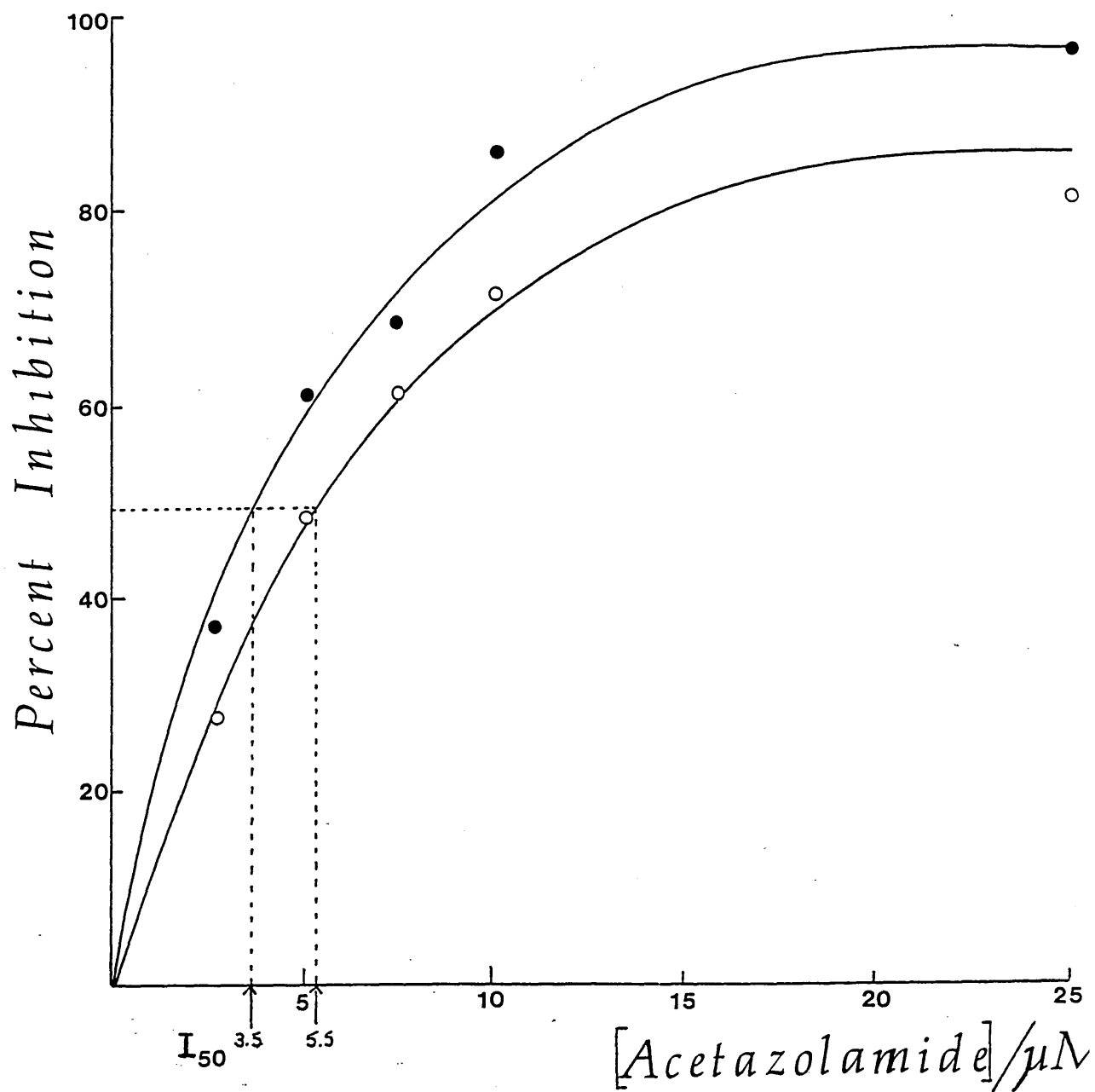
Chicken CA III was incubated in (●) 60 mM BD in 50 mM borate buffer pH 8.3 or (□) 125 mM P_i in 50 mM borate buffer pH 8.3 for 6 h and assayed in 10 mM hepes as described (2.8.1) at pH 6.7-7.5. Control enzyme incubated in 50 mM borate buffer pH 8.3 was assayed at pH 6.7-7.5 in either 10 mM hepes (▼) or 10 mM phosphate (■).

4.6.4 Acetazolamide Inhibition and the Effect of Phosphate

Investigations of acetazolamide binding in carbonic anhydrase have been extensive, but many of the muscle CA studies were conducted in phosphate buffer. A preliminary examination of the I_{50} for acetazolamide in chicken and human CA III indicated that the inclusion of phosphate reduced the level of inhibition and consequently raised the I_{50} .

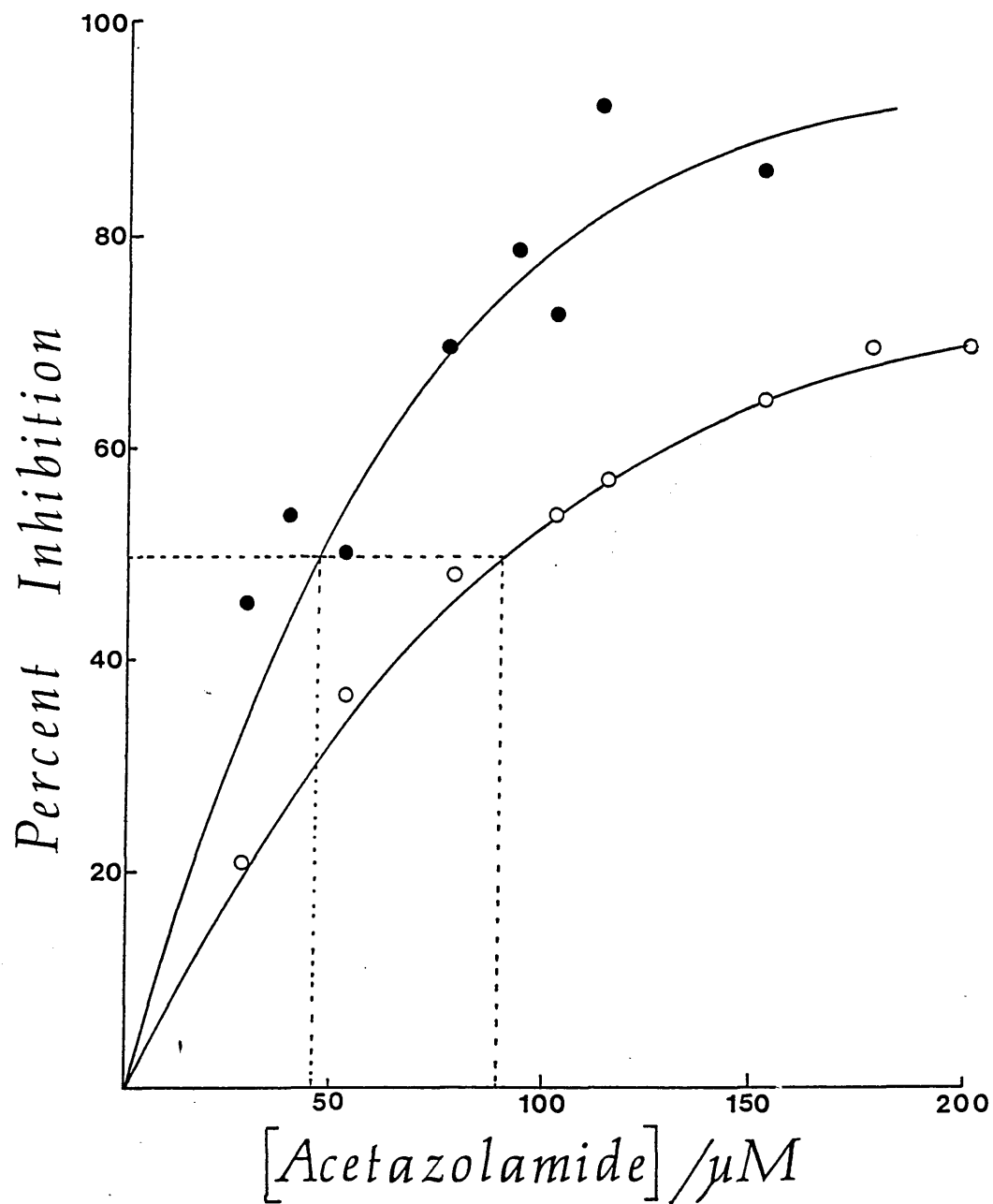
Figure 4.21 shows the pattern of inhibition by this sulphonamide in 10 mM hepes and 10 mM phosphate, for chicken CA III. A respective I_{50} of 4 μ M and 6 μ M was recorded. In the human sample, the I_{50} of 40 μ M in hepes increased to 80 μ M in phosphate (Figure 4.22).

FIGURE 4.21 Acetazolamide Inhibition of the HCO_3^- Dehydration
Activity of Chicken CA III and the Effect of Phosphate



Chicken CA III was assayed in 10 mM phosphate (○) or 10 mM hepes buffer (●) at pH 7.1, 2°C.

FIGURE 4.22 Acetazolamide Inhibition of the HCO_3^- Dehydration Activity of Human CA III in the Presence and Absence of P_i



Human CA III was assayed in 10 mM phosphate (○) or 10 mM hepes buffer (●) at pH 7.1, 2°C.

4.6.5 Anion Inhibition and the Effect of Phosphate

The chemical and kinetic nature of anion inhibition of the carbonic anhydrases has been extensively studied (Maren and Sanyal, 1983). Anion inhibition is not complicated by slow association that prevails for many sulphonamides and is thought to be classically competitive with respect to HCO_3^- dehydration, but mixed with CO_2 as substrate (Maren and Couto, 1979). Our findings for a range of anions suggests a non-competitive nature of anion inhibition for the back reaction, and two Dixon plots are included for reference (Figures 4.23, 4.24) (Shelton and Chegwiddden, 1987).

The effect of chloride inhibition was studied in hepes buffer using unmodified chicken CA III and P_i -modified conditioned enzyme (Figure 4.25). At low $[\text{Cl}^-]$ the P_i -modified enzyme exhibited some degree of activation, which gradually disappeared to be replaced by inhibition as the $[\text{Cl}^-]$ increased. Thus at 12.5 mM Cl^- , the P_i -modified enzyme is 'resistant' to this anion, whilst the unmodified protein is inhibited by 20%. There is a consequential decrease in the I_{50} from 75 mM to 65 mM. It appears that at chloride concentrations equivalent to physiological levels, previous exposure of CA III to phosphate nullifies the effect of the anion.

FIGURE 4.23 Dixon Plot Showing Inhibition of HCO_3^- Dehydration Activity of Human CA III by Sodium Chloride

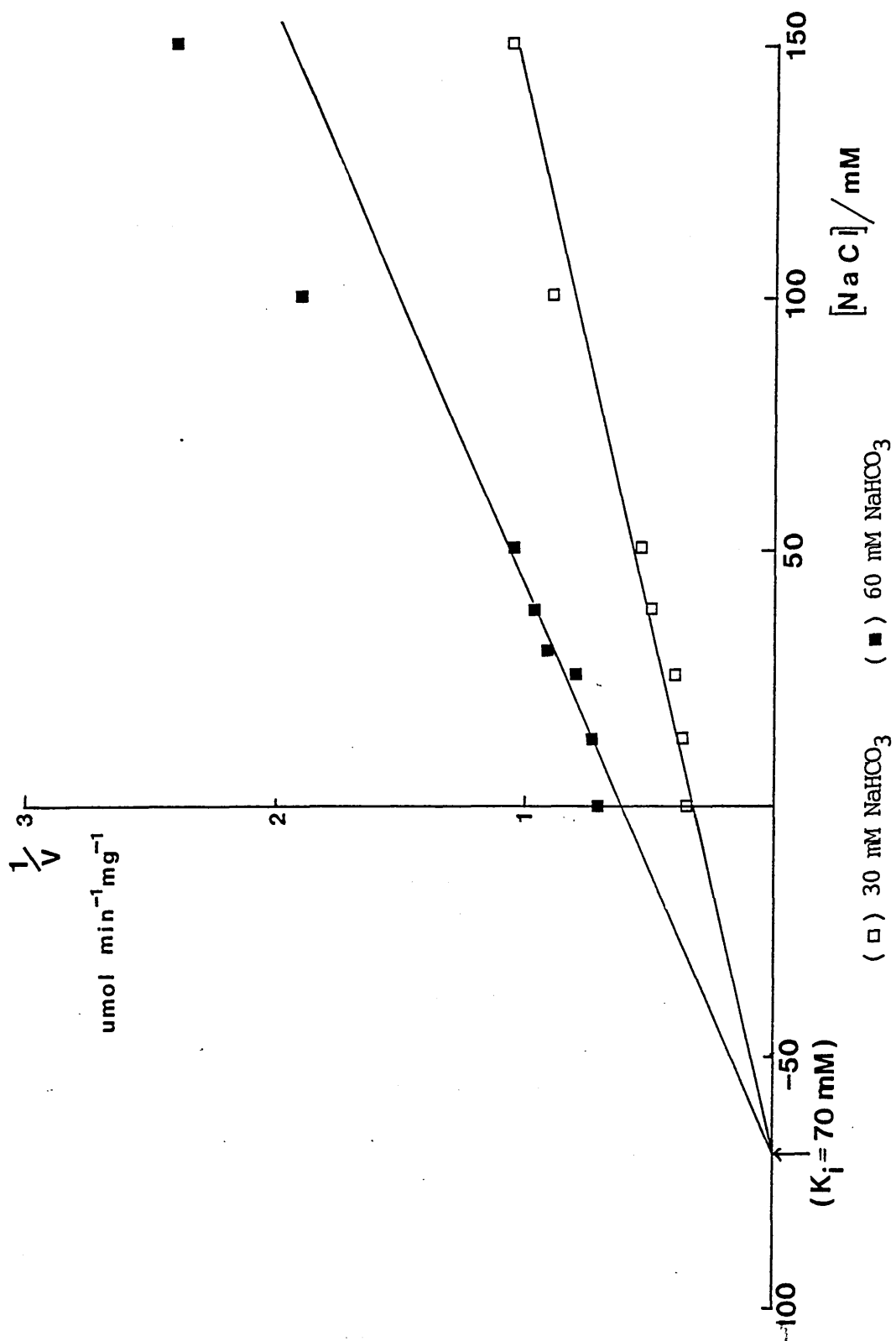


FIGURE 4.24 Dixon Plot Showing Inhibition of HCO_3^- Dehydration Activity of Chicken CA III by Sodium Chloride

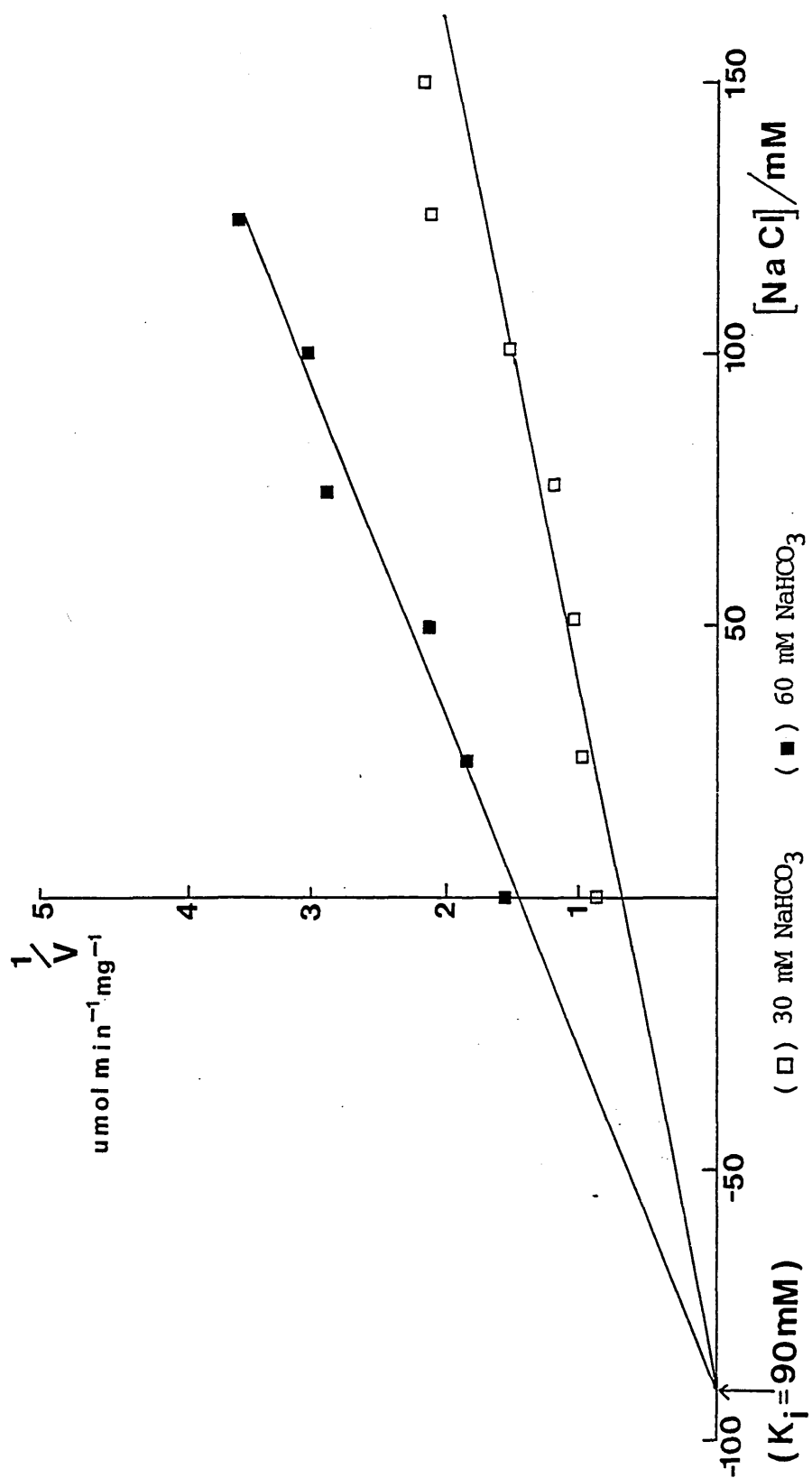
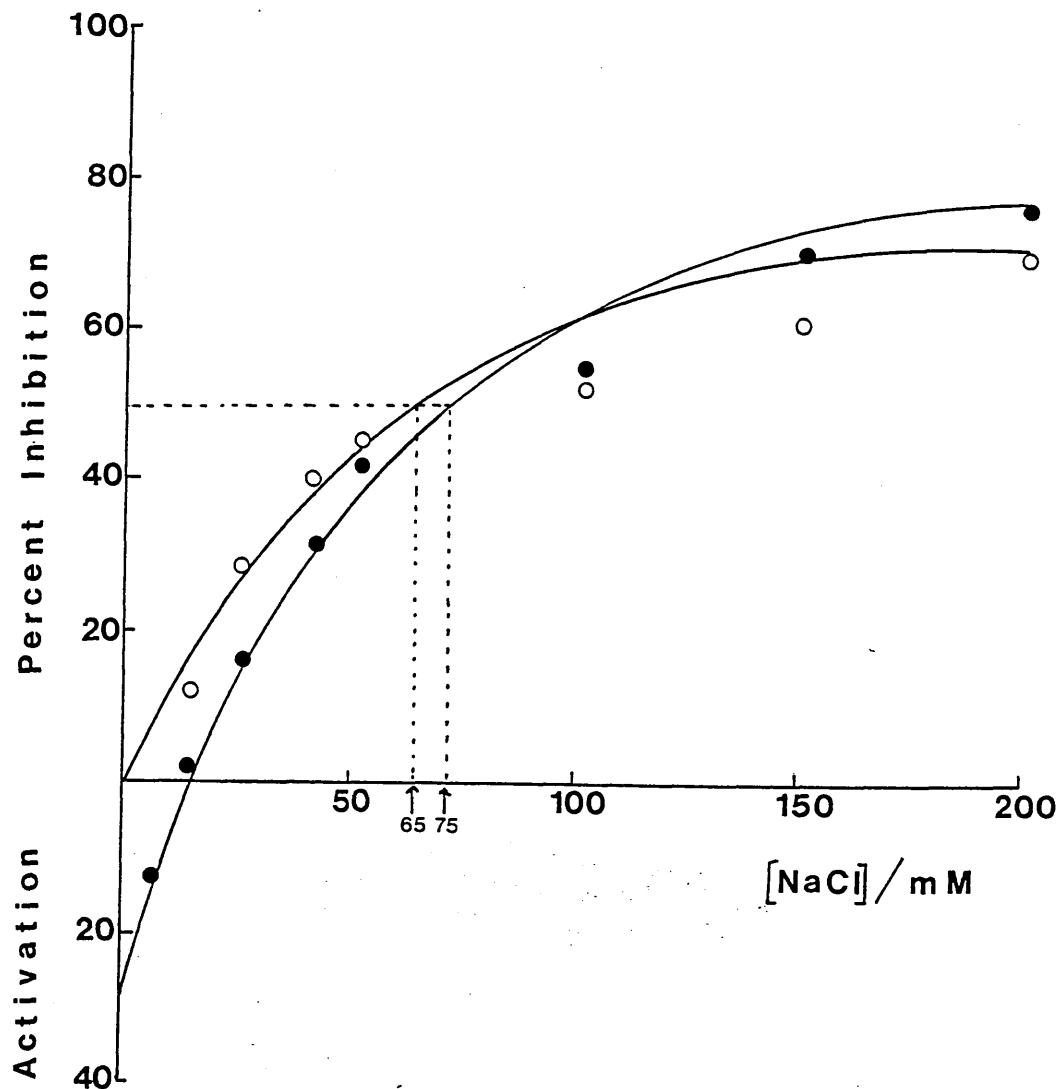


FIGURE 4.25 Inhibition of the HCO_3^- Dehydration Activity of Chicken CA III and P_i -modified Chicken CA III by Chloride Ions

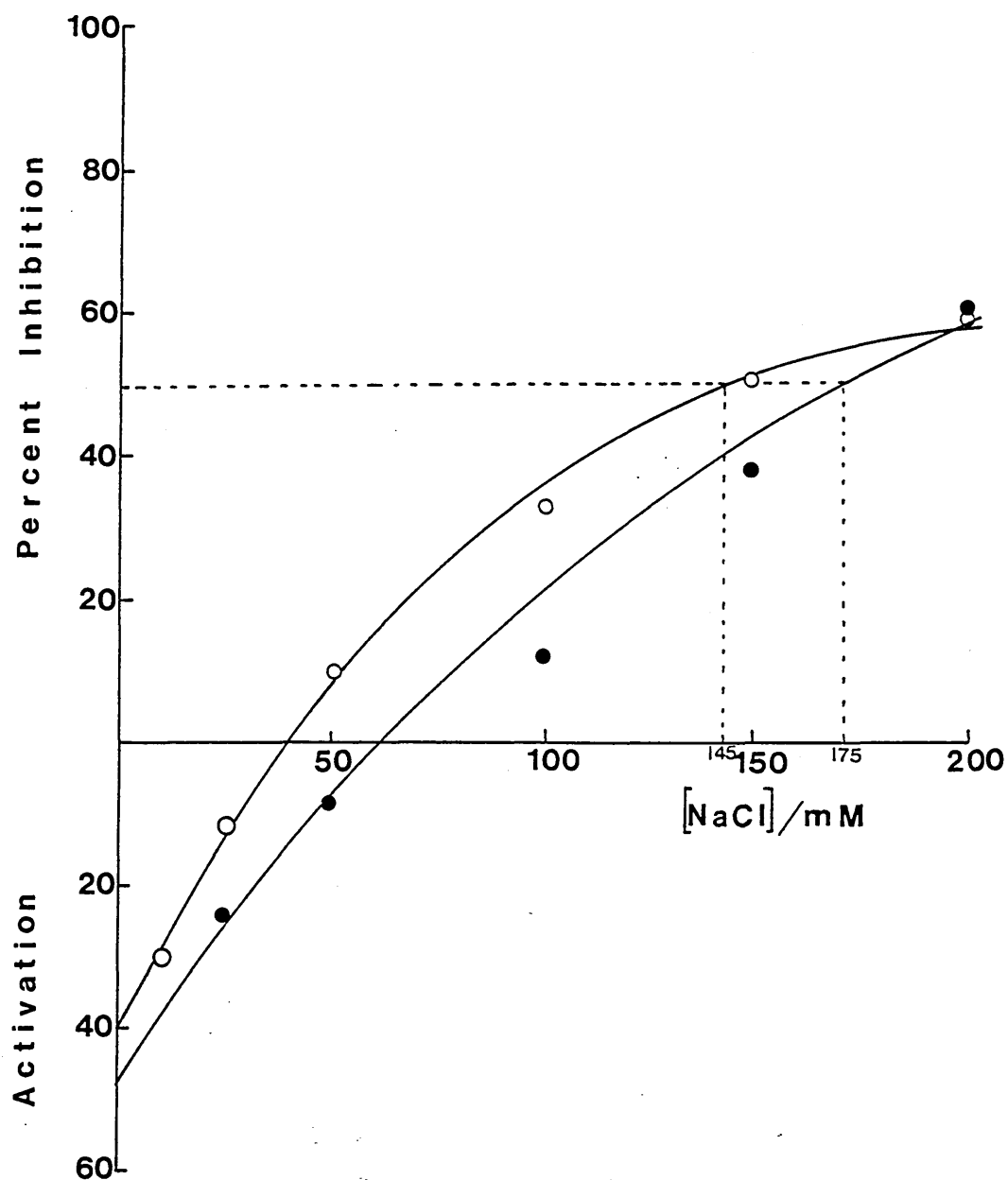


Chicken CA III was preincubated in either 125 mM P_i in 50 mM borate buffer pH 8.3 for 1 h (●) or 50 mM borate buffer pH 8.3 (○). All assays were performed in 10 mM hepes buffer pH 7.1 at 2°C, 30 mM NaHCO_3 .

The previous experiment was repeated with 10 mM phosphate buffer instead of hepes in the assay mixture. It is clear from Figure 4.26, that preincubation with phosphate plus phosphate in the medium significantly reduces chloride inhibition. Thus at 50 mM, NaCl is ineffectual as an inhibitor in the preincubated protein; this compares to 50% inhibition demonstrated by the unmodified protein in hepes (Figure 4.25).

It is unlikely that phosphate directly competes with the zinc bound HCO_3^- , although it is not inconceivable that binding of phosphate somehow promotes the rate limiting proton step or favours substrate binding. Whether or not chloride is directly involved in the phosphate activating mechanism is impossible to state. Quite simply, chloride could be inhibiting independently from phosphate activating. Whatever the mechanism, these findings may well be physiologically significant, since both chloride and phosphate play a vital role in ion transport and regulation in active muscle.

FIGURE 4.26 Inhibition of the HCO_3^- Dehydration Activity of Chicken CA III and P_i -modified Chicken CA III by Chloride and the Effect of Phosphate Ions



Chicken CA III was preincubated in either 125 mM P_i in 50 mM borate buffer pH 8.3 for 1 h (●) or 50 mM borate buffer pH 8.3 (○). All assays were performed in 10 mM phosphate buffer.

4.7 COMPARATIVE STUDIES WITH CA I

a) Effect of Anions and Phosphorylated Metabolites

The effect of phosphate on human CA I is described in section 4.2 (Figure 4.3). The study on CA I activity was extended to include the effects of anions and phosphorylated metabolites known to enhance CA III bicarbonate dehydration activity. In contrast to CA III, the addition of ATP, ADP, and HSO_3^- resulted in only a slight increase in CA I activity and 1,3-BPG was without effect.

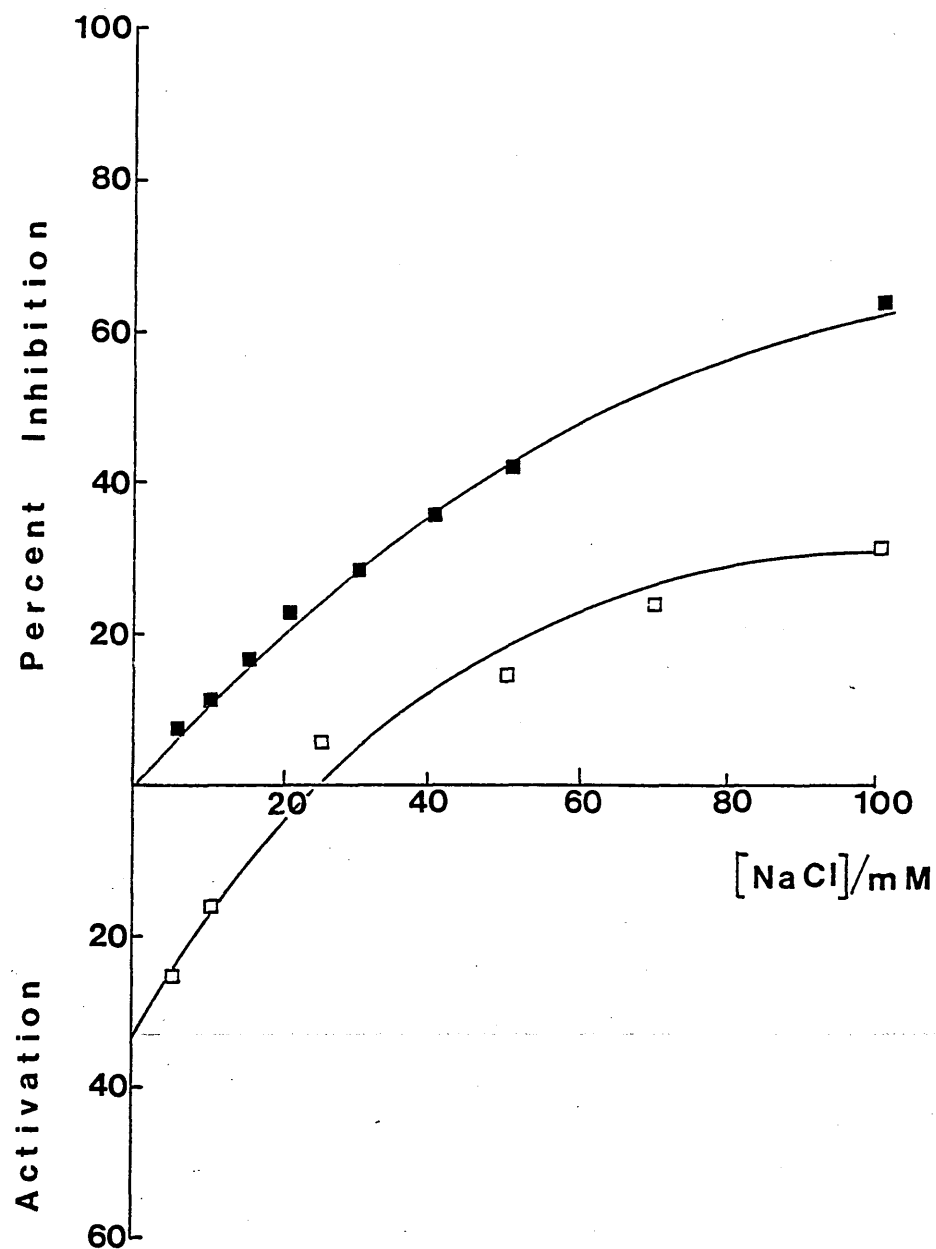
TABLE 4.8 Effect of Anions and Phosphorylated Metabolites on the HCO_3^- Dehydration Activity of Human CA I

| | Conc mM | % Activation |
|------------------|---------|--------------|
| ATP | 5 | 0 |
| | 15 | 16 |
| ADP | 5 | 10 |
| | 15 | 30 |
| cAMP | 10 | - |
| AMP | 15 | 12 |
| HSO_3^- | 10 | 29 |
| 1,3-BPG | 2.3 | - |
| P_i | 15 | 81 |

b) Chloride Inhibition

CA I is particularly sensitive to anion inhibition, in fact, Cl^- is inhibitory at a concentration found physiologically in erythrocytes (Sanyal *et al*, 1982). Maren (1967) reported a K_i for Cl^- of 50 mM for erythrocytes containing a mixture of CA I and CA II and the approximate $[\text{Cl}^-]$ of red cell fluid is 80 mM. Singly, human CA I has a K_i value of 6 mM (CO_2 hydration, 0°C, Sanyal *et al*, 1982). This sensitivity of CA I is shared by CA III for some anions (Shelton and Chegwiddden, 1987), whereas CA II is more resistant. In view of the finding that chloride inhibition is masked by P_i activation in chicken CA III, the study of Cl^- inhibition was extended to human CA I and the effect of phosphate examined. The results in Figure 4.27 indicate that phosphate also overcomes chloride inhibition in this isoenzyme.

FIGURE 4.27 Anion Inhibition of the Bicarbonate Dehydration
Activity of Human CA I and the Effect of Phosphate Ions



Human CA I assays were performed in 10 mM hepes (■) or 10 mM phosphate buffer (□).

4.8 DISCUSSION

The results presented in this section clearly indicate that phosphate enhances the HCO_3^- dehydration activity of muscle CA III. The concentration range over which these effects are observed, of between 5 and 50 mM, corresponds to the normal range of free inorganic phosphate detected in whole muscle during exercise. In normal human subjects, the resting P_i level of about 4 mM rises to about 26 mM after exercise (Bevington *et al*, 1986; Taylor *et al*, 1983; Arnold *et al*, 1985). The observed effect of activation by phosphate can be extended to some intermediates of the glycolytic pathway, adenine nucleotides and other anions. Again, the concentrations used in these studies are well within the homeostatic physiological levels present in the active muscle cell.

Exposure of chicken CA III to transient high levels of phosphate probably results in a relatively stable P_i -enzyme complex. Localised pools of phosphate are an integral feature of many active cells (Jahan and Butterworth, 1988). It is thus conceivable that CA III may be physiologically exposed to P_i concentrations in the range 25-125 mM. The ionic interaction between phosphate and chicken CA III forms rapidly, whilst subsequent dissociation and equilibration with the surrounding buffer is a slower process, requiring at least thirty minutes. This slow dissociation could sustain the P_i effect under conditions of fluctuating $[\text{P}_i]$.

Measurements of the kinetic parameters of chicken muscle CA III suggest that phosphate reduces K_M whilst k_{cat} remains constant. Since both chicken and human CA III function physiologically well below their respective K_M s, then the indices of catalytic effectiveness are represented by the apparent rate constant k_{cat}/K_M . Phosphate increases the k_{cat}/K_M of chicken CA III, suggesting that this enzyme functions more efficiently in its native surroundings than had been appreciated. Many of the kinetic constants have been measured in barbitol buffer and CA III is usually quoted as exhibiting 3% of the activity of CA II. In reality, the relative specific activity is probably higher. Studies with horse CA III indicate that under optimal conditions with buffer containing 13 mM phosphate this isoenzyme approaches CA I in terms of relative activity.

There are several reports in the literature that CA III from different species is independent of pH over a physiological range. It has been inferred that this feature of muscle CA might be of selective advantage in a tissue with dramatic pH fluctuations. Whilst the activity of chicken CA III remains virtually constant in hepes buffer between pH 6.5 and 7.5, it appears that previous exposure to P_i or the addition of P_i renders the enzyme very responsive to pH change. This sensitivity rather than conferring a deleterious effect might be of selective advantage. Fatigue is accompanied by a high $[CO_2]$ and low pH. Any enzyme equipped to respond both to the phosphorylation potential of the cell and the pH, which could rapidly restore pH homeostasis, may well be conserved. It is tempting to speculate that the requirement for prolonged performance in red skeletal muscle is greater than in fast muscle; hence the location of CA III in type I fibres.

The mechanism of phosphate-enzyme interaction is complex. Prior treatment of chicken CA III with the arginine modifying agent, 2,3-butanedione, precludes further modification by pretreatment with P_i . Yet both BD-modified and P_i -modified enzyme can be additionally activated by augmenting the medium with phosphate. This implies that BD and P_i may both bind to arginine residues in the active site, with a concomitant conformational change resulting in enhanced activity. Transfer to an environment containing phosphate increases activity by the interaction of P_i at another site or perhaps by accelerating the proton transfer step to the buffer. The fact that pretreating chicken CA III with BD followed by P_i pretreatment shows less activation than when BD and P_i are together competitively, implies that although the site of binding may be the same, the conformational change is perhaps different. Previous studies with BD indicate that a slow conformational change takes place over twenty-four hours, after an initial response. The response to phosphate is instantaneous.

A comparison of the effects of phosphate on CA I and CA III activity also suggests two modes of phosphate action. HCO_3^- dehydration of CA I is activated by phosphate, albeit to a lesser degree than that of CA III, yet it has no arginines in the active site. One can hypothesize that CA I and CA III share a secondary phosphate binding site, or a mechanism that increases catalytic efficiency, which is absent in CA II.

The effect of adding phosphoryl esters and phosphate together indicates that these metabolites/ions share a common site or activating mechanism which is fully operational in these studies. No phosphatase activity was evident from the inorganic phosphate

estimations. Though the hypothesis that the true phosphorylated substrate has yet to be identified cannot be ruled out.

Binding of phosphate to chicken CA III confers some degree of resistance to acetazolamide inhibition and considerable resistance to chloride inhibition. CA I is also resistant to chloride inhibition when phosphate is present. Chloride competes with HCO_3^- in the dehydration reaction for ligand binding to the zinc molecule. If CA I and CA III share a secondary phosphate binding site, then phosphate binding may directly alter the $\text{HCO}_3^-/\text{Cl}^-$ relationship or phosphate activation may be quite separate from chloride inhibition and the two systems are counterbalancing each other.

Whatever the mechanism of phosphate activation, there is no doubt that phosphate has a subtle and complex effect on the HCO_3^- dehydration activity of muscle carbonic anhydrase, that probably operates under physiological conditions.

5.1 INTRODUCTION

Thus far, the esterase reaction has received little attention with regard to the muscle carbonic anhydrase isoenzyme. Since the carbonic anhydrases hydrolyse a number of esters, a pilot study was undertaken to select the most suitable esterase assay.

5.2 COMPARISON OF THE ESTERASE ACTIVITIES OF CHICKEN CA III

The esterase activities of chicken CA III towards 2-NPA, 4-NPA, 2 hydroxy-5-nitro- α -toluenesulphonic acid sultone (HNTAS) and β -naphthyl acetate are shown in Table 5.1. These were determined by the methods described in section 2.8.2, and the HNTAS substrate was prepared as in the text.

There is a notable difference between 2-NPA and 4-NPA with respect to chicken CA III catalysis. No absorbance changes were observed with 2-NPA below 3 mM, whereas 4-NPA yielded accurate and reproducible results. The insolubility of 2-NPA precluded activity measurements above 3.5 mM. β -naphthyl acetate also has a low aqueous solubility. Dissolving this ester initially in a small volume of acetone appeared to interfere with the assay yielding spurious results. By contrast, CA III rapidly hydrolyses the sulphonate ester sultone, in spite of the requirement of acetone as partial solvent. Acetone was used as solvent in preference to acetonitrile to eliminate any possible enzyme inhibition by cyanide.

TABLE 5.1 Esterase Activities of Chicken CA III

| | [S] mM | Specific Activity nmol min ⁻¹ mg ⁻¹ enzyme |
|---------------------------|---------|---|
| 2-NPA | 1.0 | - |
| | 1.9 | - |
| | 3.0 | 2.1 |
| 4-NPA | 1.0 | 4.3 |
| | 1.9 | 6.1 |
| | 3.0 | 6.8 |
| HNTAS | 1.0 | $3.6 \times 10^{-3} \Delta A s^{-1} mg^{-1} enzyme$ |
| | 3.0 | $7.9 \times 10^{-3} \Delta A s^{-1} mg^{-1} enzyme$ |
| β -naphthyl acetate | 0.5-5.0 | - |

Thus, 4-NPA and HNTAS were selected for kinetic study and for investigating the effects, if any, of phosphate, phosphorylated metabolites and BD-modification on the esterase reaction. Data for CA I and CA II are included where appropriate.

5.3 4-NITROPHENYL ACETATE HYDROLYSIS

5.3.1 Kinetic Studies of the Carbonic Anhydrase Isoenzymes

The K_M values and the k_{cat}/K_M ratios for chicken CA III, human CA I, CA II and III are shown in Table 5.2 (25°C, pH 7.1). In contrast to the HCO_3^- -dehydration reaction, the determination of these kinetic parameters for the esterase activity of chicken CA III was experimentally less intricate. The insolubility of 4-NPA precluded activity measurements above 3.75 mM, but traces below this

concentration were sufficiently accurate to permit determination of K_M and V_{max} . A typical Michaelis-Menten curve for chicken CA III is included (Figure 5.1). Each set of data were analysed by straight line plots, which were subsequently compared to the parameters obtained using the Enzfitter computer programme based on the Michaelis-Menten equation (section 2.12). The curve illustrated in Figure 5.1 is shown as a Lineweaver-Burk plot (Figure 5.2), a Hanes plot (Figure 5.3) and a Eadie-Hofstee plot (Figure 5.4). There is good agreement between the different mathematical treatments (Table 5.4). The K_M and k_{cat}/K_M values quoted in Table 5.2 were calculated from eight sets of data.

TABLE 5.2 Comparison of the Kinetic Parameters of Carbonic Anhydrase Isoenzymes Towards 4-NPA

| | K_M mM | k_{cat} s ⁻¹ | k_{cat}/K_M M ⁻¹ s ⁻¹ |
|----------------|-------------|---------------------------|--|
| Chicken CA III | 1.7 (± 0.5) | 8 | 5 |
| Human CA I | 46 | 24500 | 530 |
| Human CA II | 10 | 36000 | 3600 |

The Michaelis-Menten curves for human CA I and CA II are shown in Figures 5.5 and 5.6. These curves were unsuitable for straight line manual plots and the parameters were calculated by computer; the high standard errors, however, indicated a considerable degree of variance. Determination of K_M and V_{max} values for human CA III was impossible due to the low solubility of 4-NPA and as illustrated in Figure 5.7, first order kinetics were evident up to 3.75 mM [S].

The relative esterase activities towards 4-NPA of CA I, and CA III isoenzymes from a number of sources are shown in Table 5.3. CA I and CA III activities are documented as a proportion of CA II activity for that species. Chicken CA III is compared to human CA II as no data is available for chicken CA II esterase activity. With the exception of the data from this thesis all the activity measurements are initial velocity measurements. Koester et al (1980) recorded that, for rabbit CA III, initial velocities rather than v values were reported because of the poor reliability of K_M determinations in the esterase reaction at all pH values. This is at variance with the consistent measurements recorded in this laboratory for chicken CA III. Nonetheless, the pattern is constant, that the muscle isoenzymes exhibit a very low esterase activity towards 4-NPA. When chicken CA III is compared to human CA II, it has 0.1% of the activity.

TABLE 5.3 Relative Catalytic Activity Towards 4-NPA Hydrolysis of Carbonic Anhydrase Isoenzymes from Various Sources (25°C)

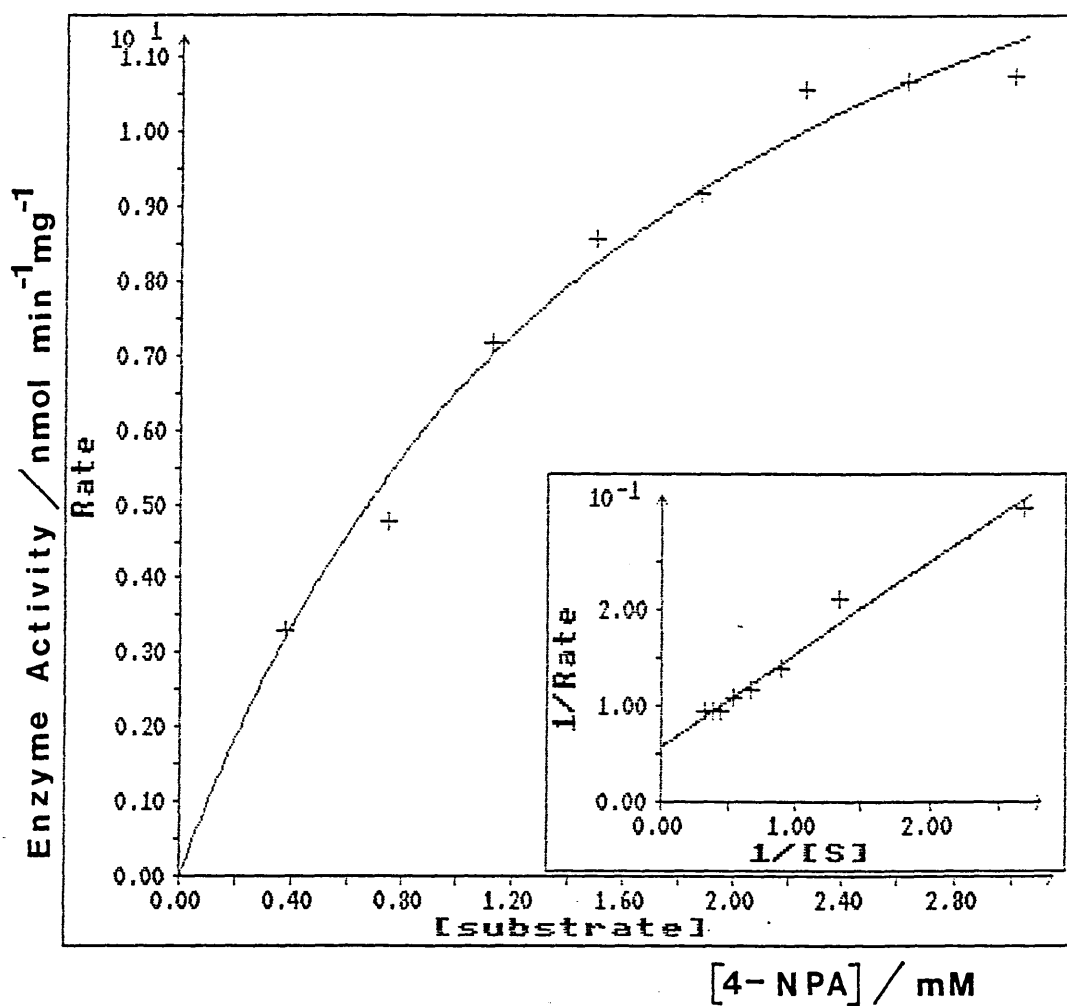
| | HUMAN | CHICKEN (compared to human) | HUMAN ¹ | BOVINE ² | RABBIT ³ |
|--------|-------|-----------------------------------|--------------------|---------------------|---------------------|
| CA I | 0.16 | - | 0.20 | | 0.12 |
| CA III | | 0.0008 | 0.0002 | 0.001 | 0.0006 |

1 Tashian et al, 1983

2 Engberg et al, 1985

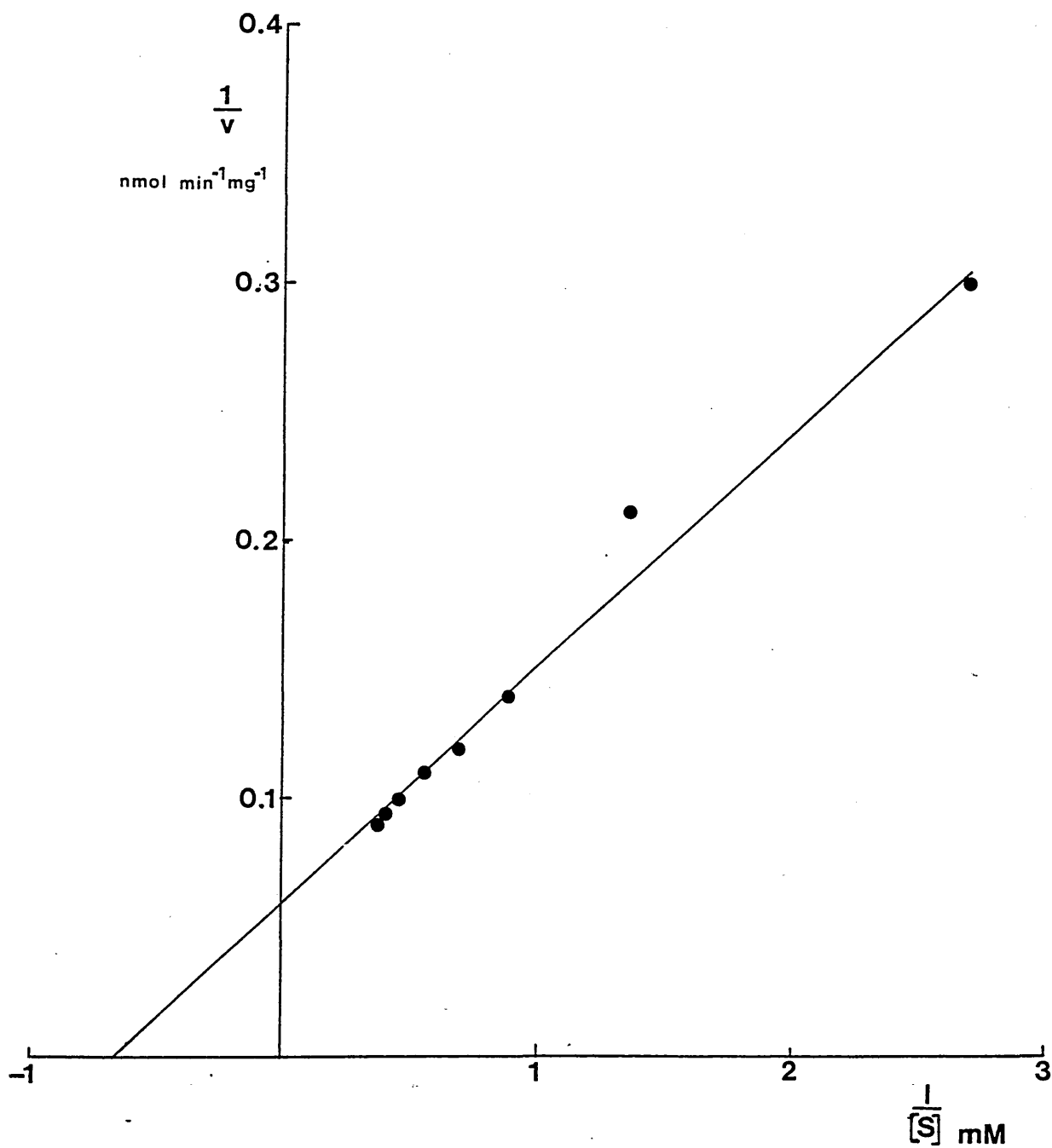
3 Koester et al, 1980

FIGURE 5.1 Computer Michaelis-Menten Curve for the Hydrolysis of 4-NPA by Chicken CA III



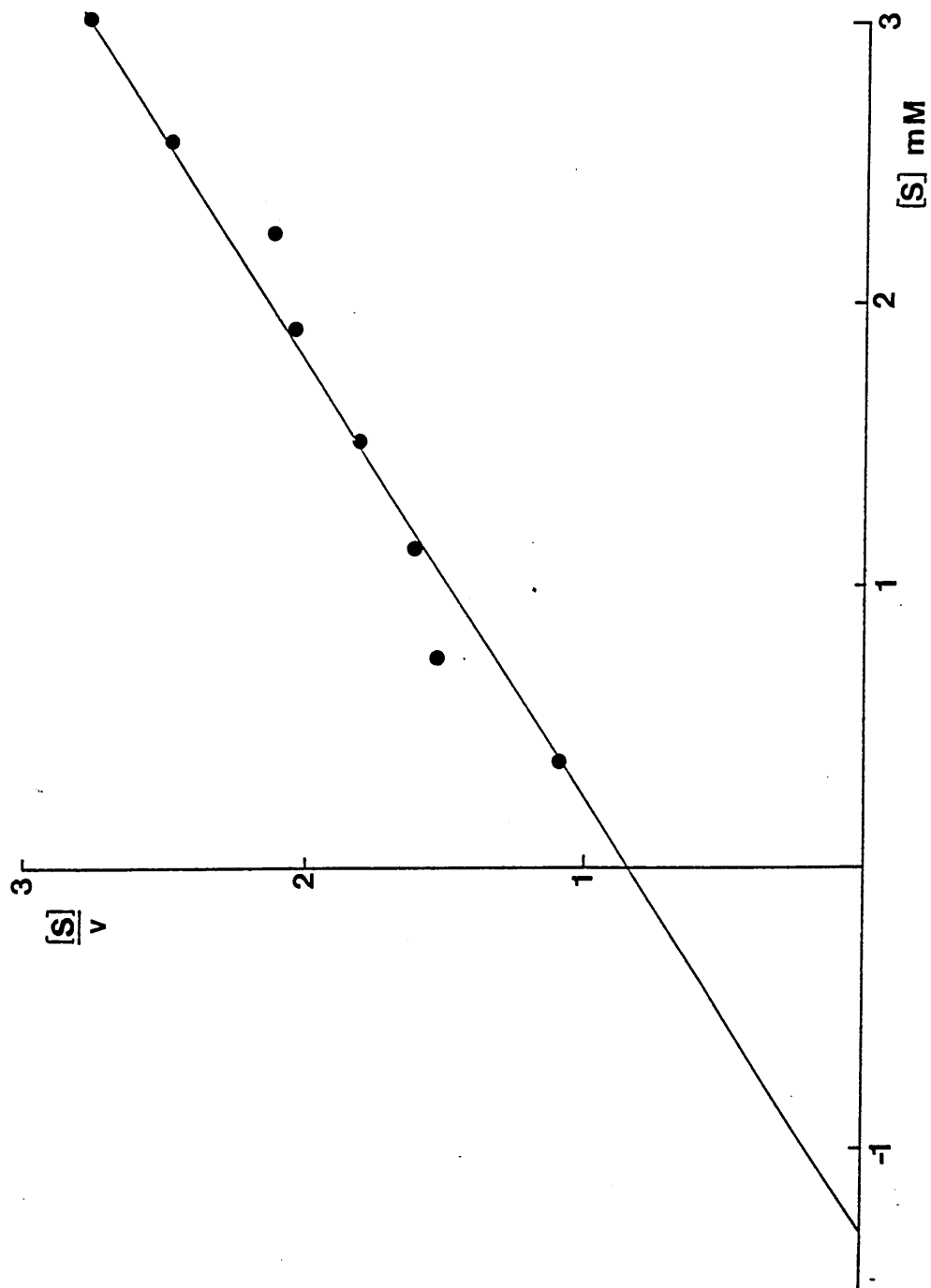
4-NPA hydrolysis was assayed as described in section 2:8.2 at 25°C and pH 7.1.

FIGURE 5.2 Lineweaver-Burk Plot of the Hydrolysis of 4-NPA by Chicken CA III



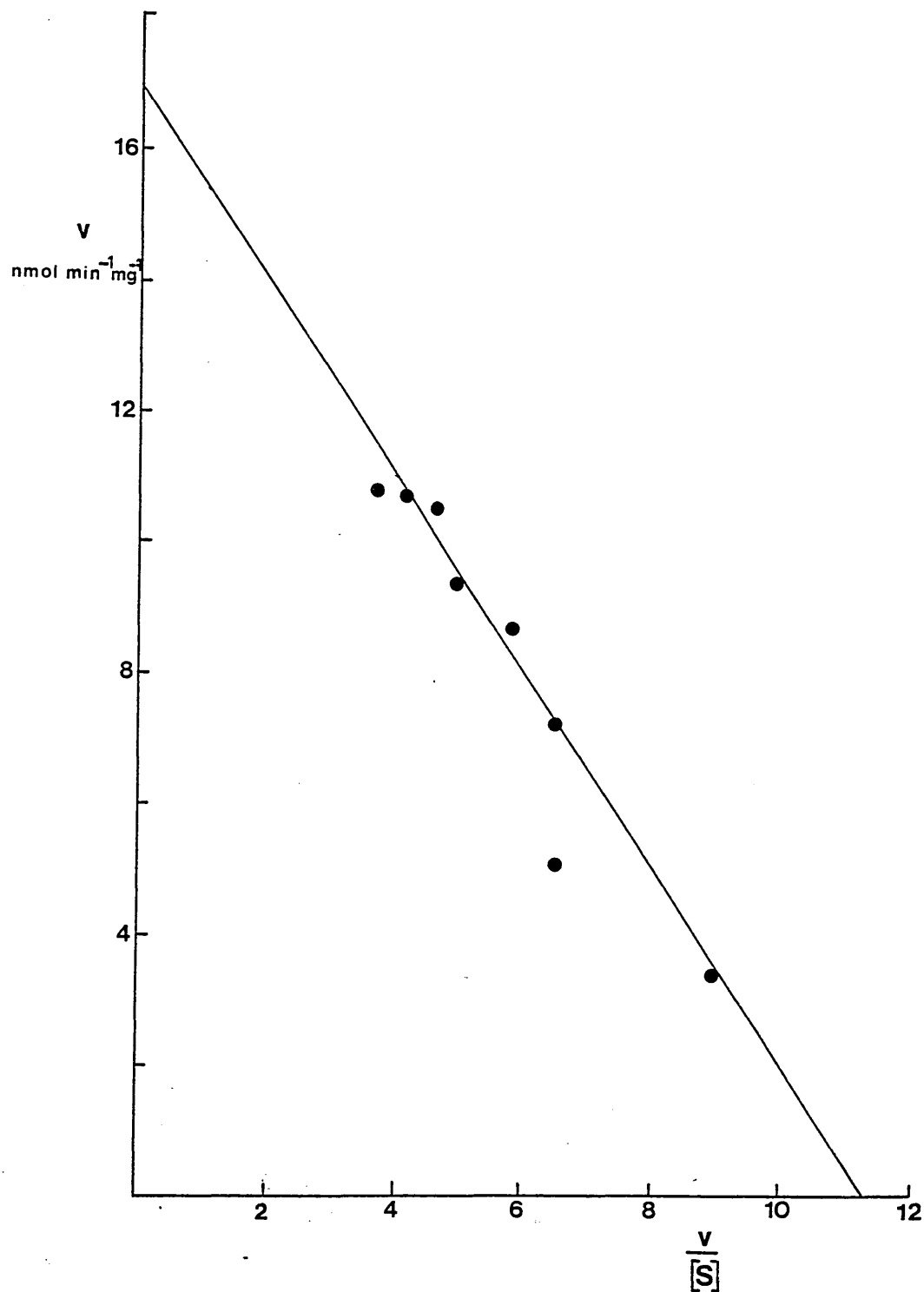
Esterase activity towards 4-NPA was measured as described in section 2.8.2. $K_M = 1.49 \text{ mM}$ $k_{\text{cat}} = 8.1 \text{ s}^{-1}$

FIGURE 5.3 Hanes Plot for the Esterase Activity Towards 4-NPA by Chicken CA III



Esterase activity towards 4-NPA was measured as described (2.8.2.)
 $K_M = 1.25 \text{ mM}$ $k_{\text{cat}} = 7.6 \text{ s}^{-1}$

FIGURE 5.4 Eadie-Hofstee Plot for the Hydrolysis of 4-NPA by Chicken CA III



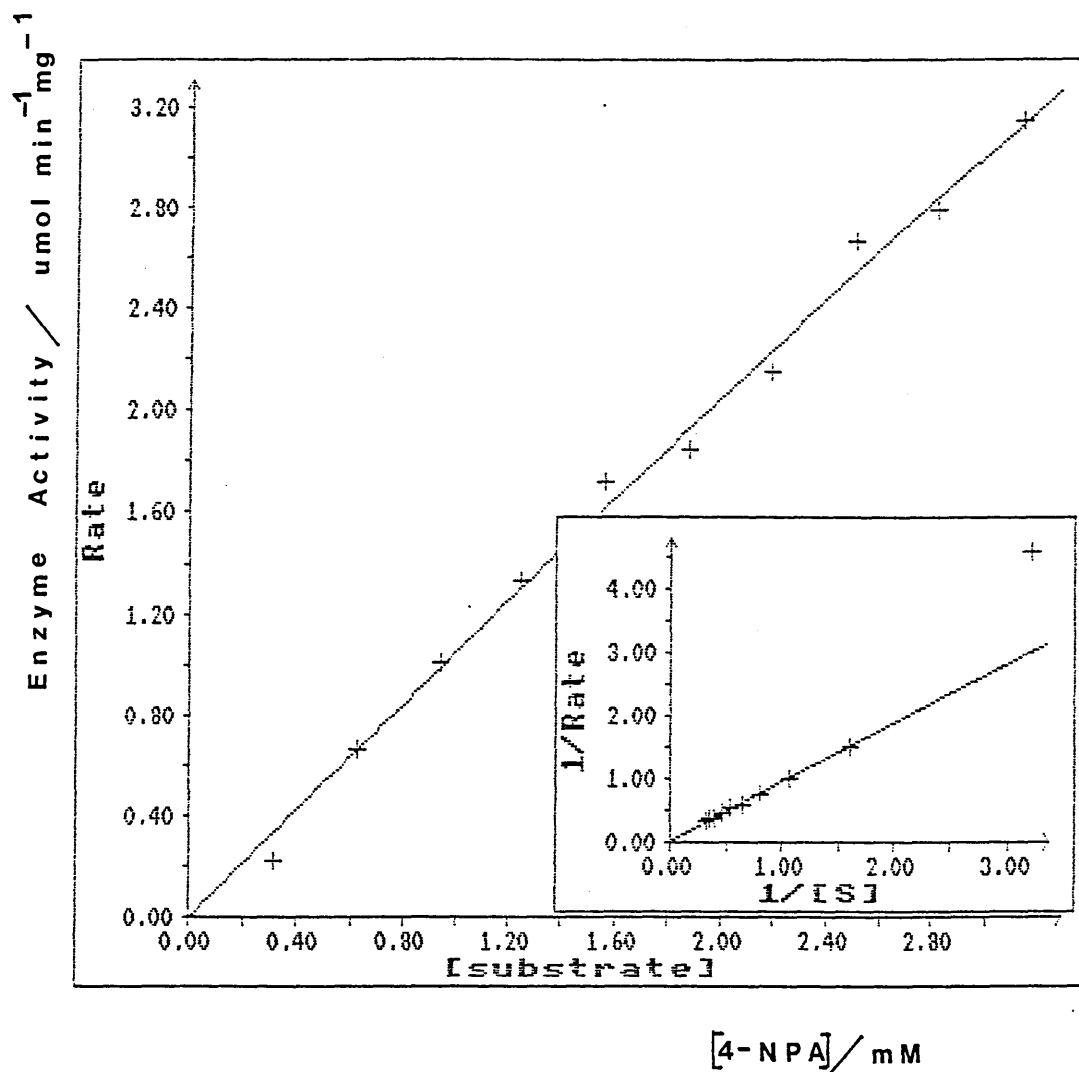
4-NPA hydrolysis was monitored as described in section 2.8.2.
 $K_M = 1.6 \text{ mM}$ $k_{\text{cat}} = 8.4 \text{ s}^{-1}$

TABLE 5.4 Comparison of the Kinetic Parameters for the Hydrolysis of 4-NPA by Chicken CA III Using Different Straight Line Plots

| | K_M mM | k_{cat} s ⁻¹ | k_{cat}/K_M M ⁻¹ s ⁻¹ |
|---|----------|---------------------------|---|
| Michaelis-Menten curve Hyperbolic regression | 1.7* | 8.7 | 5.2 |
| Lineweaver-Burk Plot | 1.5 | 8.1 | 5.4 |
| Hanes Plot | 1.3 | 7.6 | 6.1 |
| Eadie-Hofstee Plot | 1.6 | 8.4 | 5.4 |

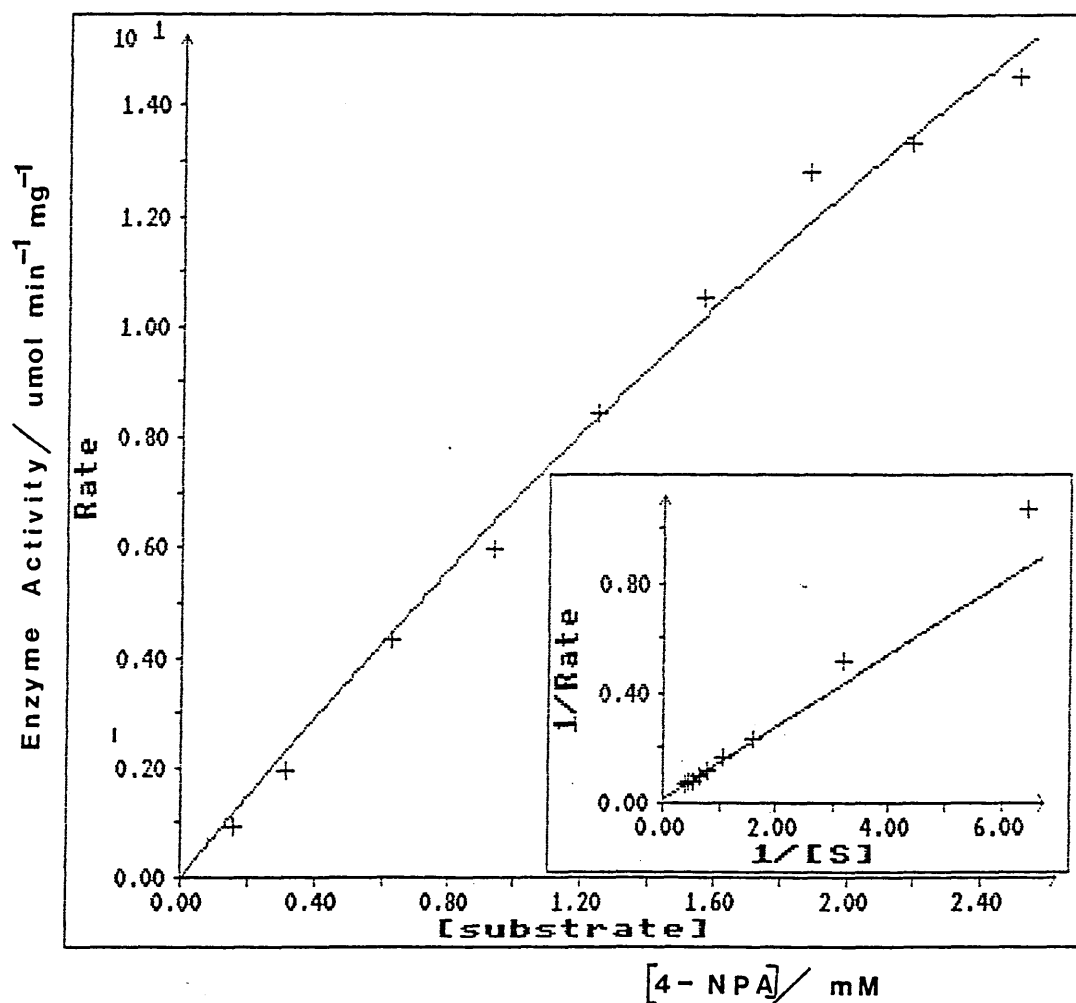
* Standard Error of the Mean 0.29

FIGURE 5.5 Michaelis-Menten Curve and Lineweaver-Burk Plot for the Hydrolysis of 4-NPA by Human CA I



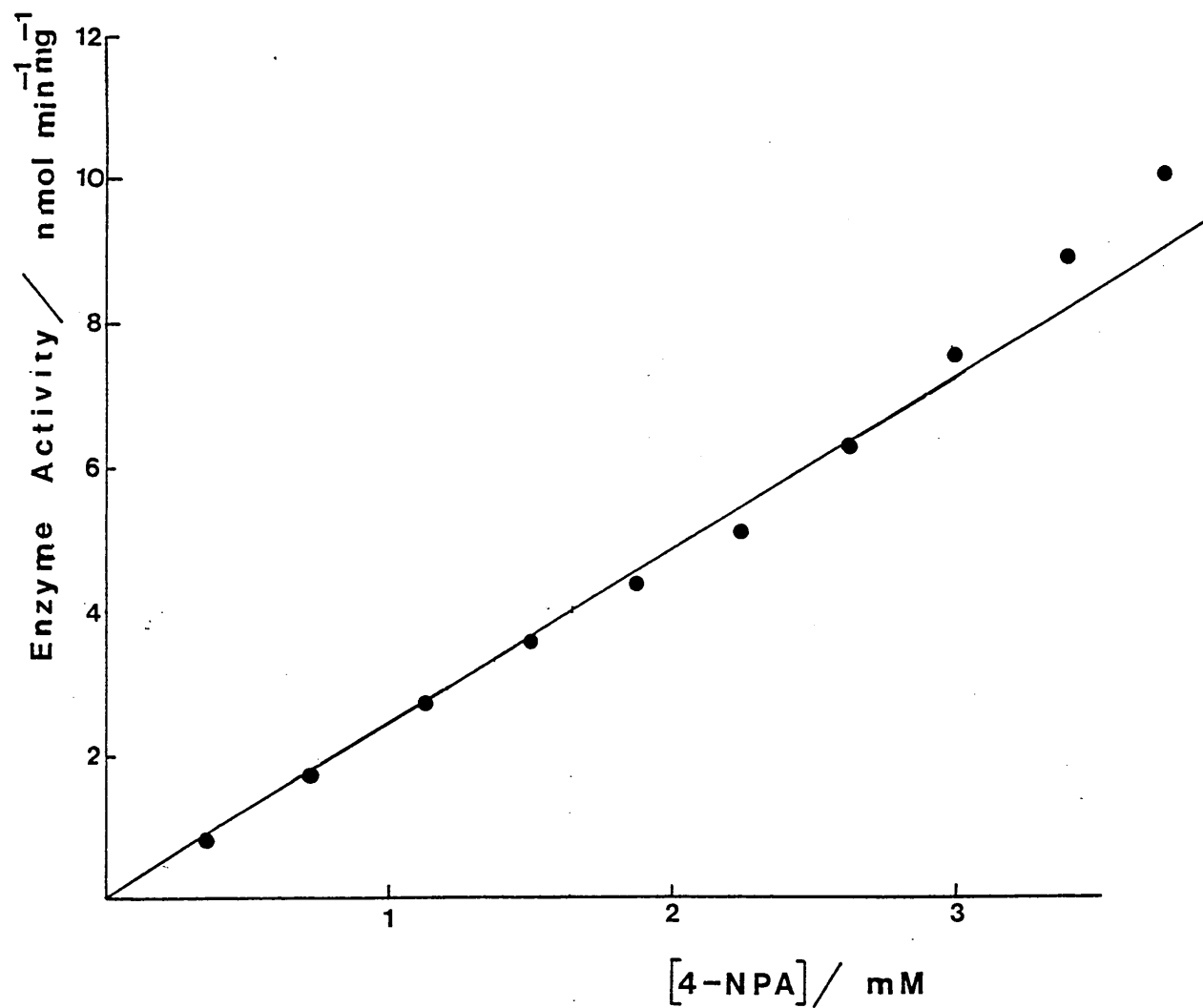
4-NPA hydrolysis was monitored at 25°C and pH 7.1. Computer analysis of the data indicated $K_M = 46 \text{ mM}$ and $k_{\text{cat}} = 24 \times 10^3 \text{ s}^{-1}$

FIGURE 5.6 Michaelis-Menten Curve and Lineweaver-Burk Plot for the Hydrolysis of 4-NPA by Human CA II



Computer analysis of the human CA II data:
 $K_M = 9.5 \text{ mM}$ (SE = 3.71); $k_{\text{cat}} = 36 \times 10^3 \text{ s}^{-1}$ (SE = 90)

FIGURE 5.7 Michaelis-Menten Curve for the Esterase Activity Towards 4-NPA by Human CA III



Esterase activity towards 4-NPA was measured as described (2.8.2) at 25°C and pH 7.1.

5.3.2 Effect of Anions, Phosphorylated Esters and Active Site
Modification on the Activity and Kinetic Parameters of
Chicken CA III.

The consistency of kinetic data recorded for chicken CA III provided an ideal model system for examining the effects of various metabolites and anions. However, daily inconsistencies can occur and effects were always correlated with control measurements recorded during the same experimental session.

a) Addition of Phosphate

Phosphate has no effect on specific activity measurements up to 27 mM at saturating substrate concentrations (Table 5.5). Above 27 mM, there is an indication that phosphate may be slightly inhibitory.

TABLE 5.5 Effect of Phosphate on the 4-NPA Esterase Activity of Chicken CA III

| $[P_i]$ mM | Specific Activity $\text{nmol min}^{-1} \text{mg}^{-1}$ |
|------------|---|
| 3.1 | 12.2 |
| 6.3 | 11.9 |
| 12.5 | 11.9 |
| 15.6 | 11.0 |
| 21.9 | 11.3 |
| 27.3 | 11.3 |
| 31.3 | 10.2 |
| 46.9 | 9.5 |

A slight effect was recorded for K_M and k_{cat} values when 15 mM phosphate was included in the esterase assay. The kinetic parameters are listed in Table 5.6.

TABLE 5.6 Effect of Phosphate on the Kinetic Parameters of the 4-NPA Esterase Reaction of Chicken CA III

| | K_M mM | k_{cat} s ⁻¹ | k_{cat}/K_M M ⁻¹ s ⁻¹ |
|------------------------|------------|---------------------------|---|
| Control | 1.7 ± 0.49 | 8.8 | 5.2 |
| 15.6 mM P _i | 1.1 ± 0.42 | 7.0 | 6.3 |

Preincubation of chicken CA III in 100 mM phosphate, contained in 5 mM Tris buffer, prior to measuring 4-NPA hydrolysis had little effect on esterase activity.

b) Effect of 1,3-Bisphosphoglyceric Acid

In contrast to the activating effect of 1,3-BPG on the bicarbonate dehydration reaction, this glycolytic pathway intermediate has no effect on the 4-NPA esterase activity. It is evident from Table 5.7 that the addition of 9.4 mM 1,3-BPG has no significant effect on the K_M and k_{cat}/K_M values of chicken CA III.

TABLE 5.7 Effect of 1,3-BPG on the Kinetic Parameters of the 4-NPA Esterase Activity of Chicken CA III

| | K_M mM | k_{cat} s ⁻¹ | k_{cat}/K_M M ⁻¹ s ⁻¹ |
|------------------|----------|---------------------------|---|
| Control | 2.2 | 6.2 | 2.9 |
| 1,3-BPG (9.4 mM) | 2.5 | 6.4 | 2.6 |

c) 2,3-Butanedione Modification

Previous studies in this laboratory have shown that 2,3-BD treatment modifies active site arginine residues and results in activation of the 4-NPA hydrolysis reaction (Chegwidden et al, 1989; Tashian et al, 1984). Using conditions known to modify arginine residues (section 2.10), the kinetic parameters were determined and are recorded in Table 5.8. Each comparative study indicated that modifying arginine residues has no effect on K_M but increases k_{cat} . Thus k_{cat}/K_M is increased by up to 50%.

TABLE 5.8 Effect of BD-Modification on the Kinetic Parameters of the 4-NPA Esterase Reaction of Chicken CA III

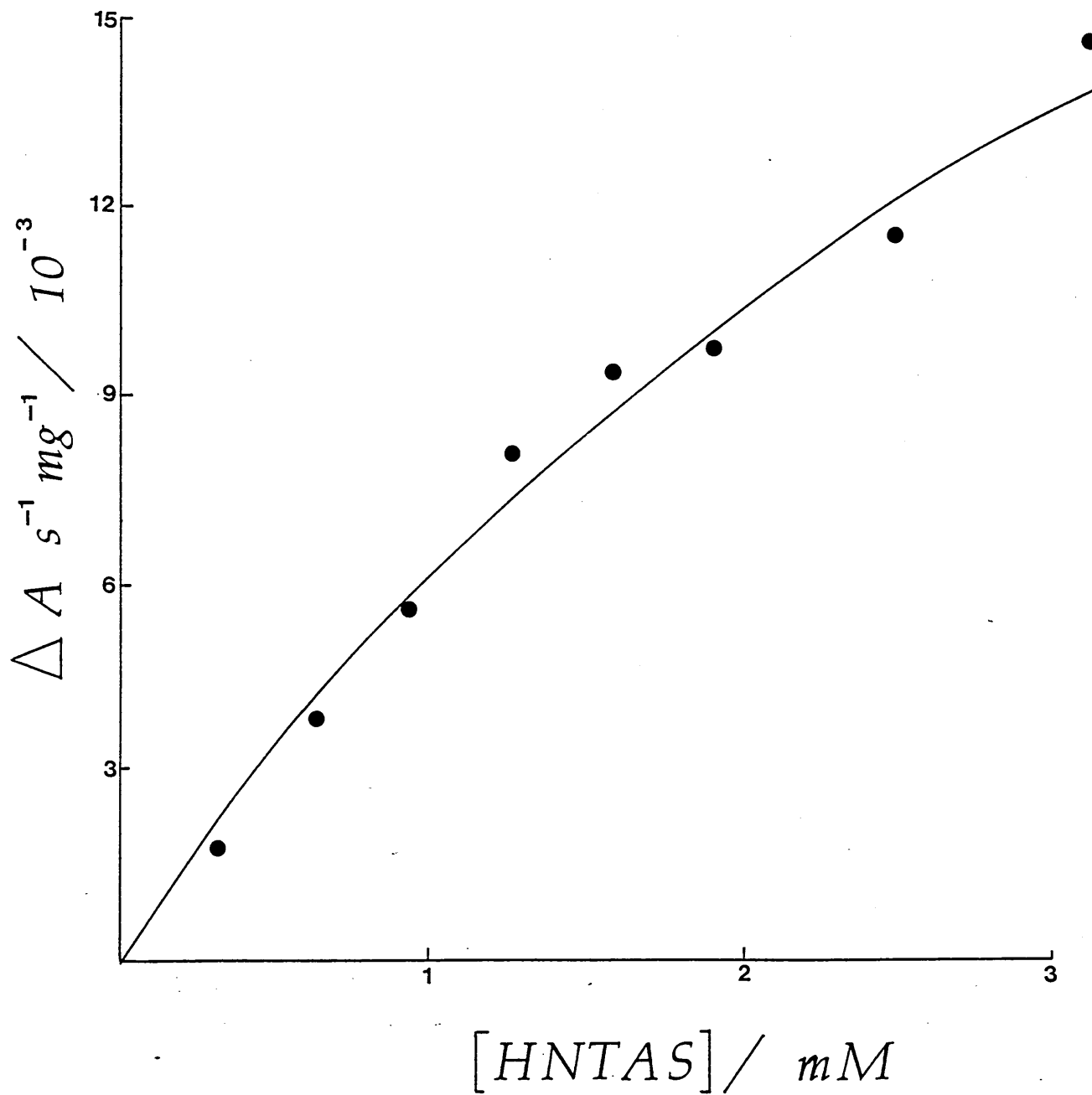
| | K_M mM | k_{cat} s ⁻¹ | k_{cat}/K_M M ⁻¹ s ⁻¹ |
|-------------|------------|---------------------------|---|
| Control | 1.4 ± 0.31 | 7.1 | 4.9 |
| BD-modified | 1.4 ± 0.28 | 10.4 | 7.2 |

5.4 HYDROLYSIS OF HNTAS

5.4.1 Kinetic Studies of Chicken CA III

The hydrolysis of HNTAS by chicken CA III was measured as described in section 2.8.2 (4) using the substrate prepared in the laboratory. The kinetics for the reaction are shown in Figure 5.8. At high substrate concentrations, the time for completion of the reaction was 30-60 seconds, zero-order kinetic measurements were determined from a tangent of initial velocity.

FIGURE 5.8 Michaelis-Menten Curve for the Hydrolysis of HNTAS by Chicken CA III



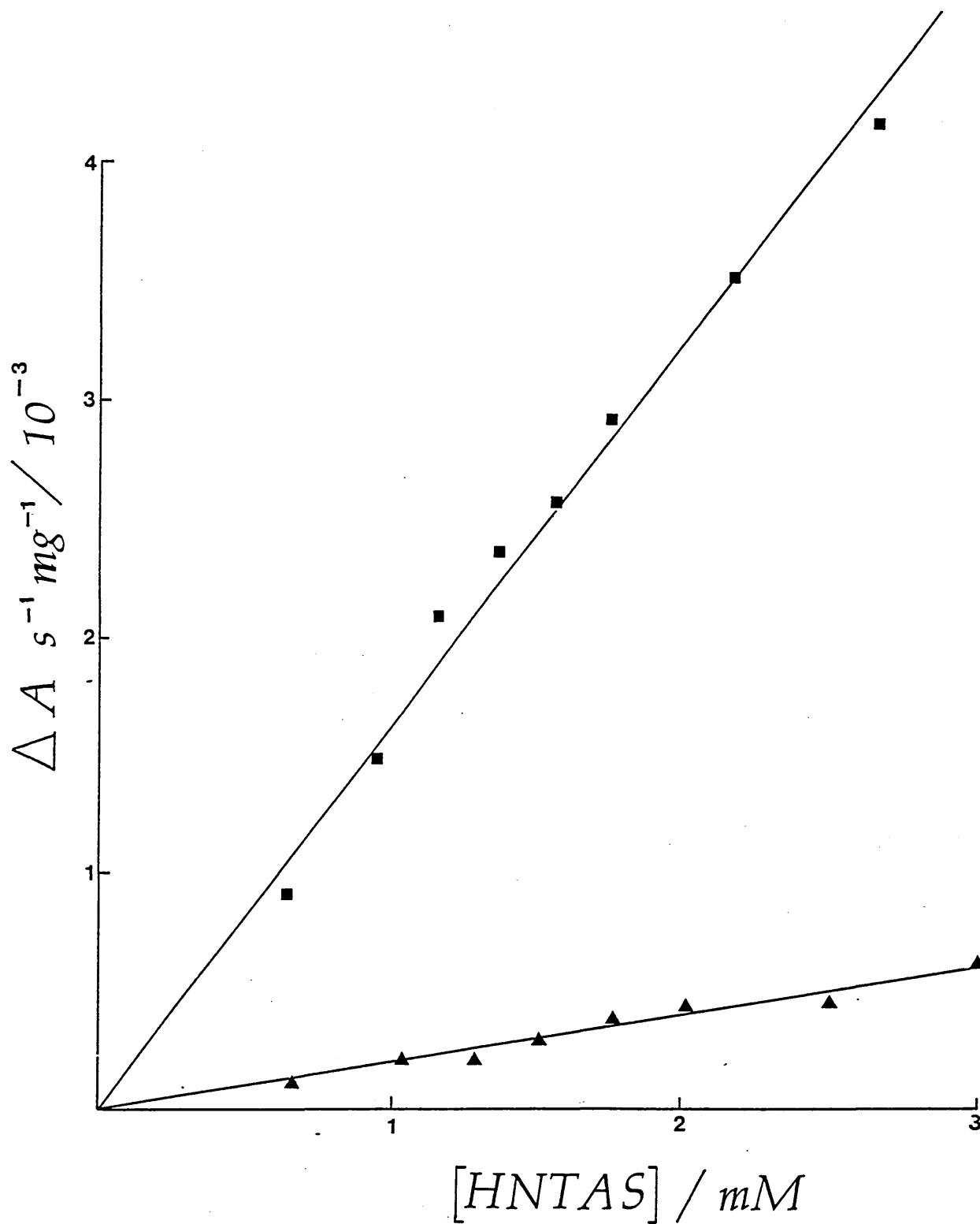
Hydrolysis of HNTAS was measured as described in 2.8.2 (4) at 25°C.
 $[E] = 2.3 \mu\text{M}$ $V_{\text{max}} = 38.1 \times 10^{-3} \Delta A \text{ s}^{-1} \text{ mg}^{-1}$ $K_M = 5.25 \text{ mM}$.

5.4.2 Effect of P_i , Anions and BD-modification

Phosphate and hydrogen bisulphite were included in the assay to monitor the effects of these anions on the catalytic activity of chicken CA III towards HNTAS hydrolysis. It was apparent that the dual-beam Carey spectrophotometer was unable to counteract the high blank value at higher substrate values. Further investigation indicated that the substrate was unstable and autohydrolysis was occurring in the presence of these ions.

The increase in absorbance with increasing concentrations of phosphate and HSO_3^- is illustrated in Figure 5.9. HNTAS was equally unstable with 2,3-butanedione. In view of the instability of this ester, persuance of kinetic determinations was limited.

FIGURE 5.9 Autohydrolysis of HNTAS in the Presence of Phosphate and Hydrogen Sulphite



Hydrolysis of sultone was measured in 15 mM phosphate (■) and 1.5 mM HSO_3^- (▲) at 25°C, pH 7.6

5.5 DISCUSSION

The catalytic activity of chicken CA III towards several esters was compared in order to identify a suitable system for investigating the effects of phosphate, other anions and BD. The aqueous insolubility of β -naphthyl acetate and low esterase activity of chicken CA III towards 2-NPA eliminated these substrates as suitable experimental systems. Additionally, HNTAS was excluded when it became apparent that this substrate was autohydrolysed by phosphate, hydrogen sulphite and 2,3-butanedione. No reference to the instability of HNTAS in particular environments could be found in the literature (Sanyal *et al*, 1983; Kaiser and Lo, 1969; Zaborsky and Kaiser, 1966).

In contrast to the aforementioned systems, the hydrolysis of 4-NPA provided an ideal reproducible model system; it produced interesting unique data with regard to the esterase activity of this avian isoenzyme.

In accordance with published data on the relative activities of the carbonic anhydrases towards 4-NPA hydrolysis, chicken CA III has a very low activity (Tashian *et al*, 1983; Engberg *et al*, 1985). Admittedly no activity measurements of chicken CA I and CA II are available for comparison, but chicken CA III has only 0.1% of the activity of human CA II, as determined under the same experimental conditions.

The results of previous studies indicate that muscle carbonic anhydrases from several species have separate sites for different substrates and thus activators/inhibitors of one activity do not perturb another activity (Nishita and Deutsch, 1985; Engberg *et al*, 1985)). Conversely, CA I and CA II share common features with regard

to CO_2 hydration and esterase activities. The physiological significance of this finding remains obscure, although it emphasises the potentially unique role of the type III isoenzymes.

The premise that the esterase and CO_2 hydration sites are physically separated in the CA III isoenzymes is partially, though not wholly, substantiated by the results presented in this section. The response of chicken CA III to phosphate and phosphorylated metabolites has been convincingly demonstrated for the HCO_3^- dehydration activity (section 4); yet phosphate and BPG are without significant effect on the hydrolysis of 4-NPA. k_{cat} and K_M remain essentially constant when P_i is included in the esterase assay at concentrations and at a pH producing a significant effect on HCO_3^- dehydration activity. These studies would apparently indicate that the esterase and HCO_3^- dehydration activities are located at separate sites on the protein.

However, the results for BD-modification of chicken CA III were equivocal. Modification of the arginine residues of chicken CA III by BD results in 50% increase in k_{cat} but K_M remains essentially constant for the hydrolysis of 4-NPA. It is worth noting that BD-modification also activates the HCO_3^- dehydration activity of this isoenzyme, although the specific effects on the kinetic parameters are undefined. Thus, the affinity of CA III for 4-NPA is unaffected by BD-modification, whereas arginine modification by BD alters the catalytic process. The BD is covalently linked to arginine residues, and the BD-arginine complex is stabilised by a bulky borate ion. It could be postulated that at pH 7.1, the borate is ionised and can thus participate in the proton shuttle catalytic mechanism without affecting 4-NPA binding. So the physical separation of the two

catalytic sites is not necessarily precluded by these studies on BD-modification, but clearly the multi-site nature of this enzyme may be an oversimplification.

It has been suggested that BD-modification of arginine residues occurs in two stages. An initial rapid modification is followed by a slower conformational change over a twenty-four hour period. In the experiments presented in this thesis, chicken CA III was modified by BD over a 1-6 hour period. Since the studies in section 4 indicate that P_i and BD show certain similarities in their mode of modification, the hydrolysis of 4-NPA by chicken CA III was also measured after prior incubation for 4 hours with P_i . Although not statistically significant, it is interesting to note that in some assays an increase in activity was observed in the P_i -modified samples. So whilst it is clear that phosphate does not significantly affect esterase activity towards 4-NPA, there remains a doubt that the esterase and CO_2 hydration sites are independent in this isoenzyme.

The kinetic and activity studies, presented in this Thesis, have centred on three main areas of research. The major area of investigation has focused on the physiologically important bicarbonate dehydration reaction in relation to the effect of inorganic phosphate, phosphorylated metabolites and various modifiers. A second line of research involved a parallel study on the esterase activities of CA III and the affects of the aforementioned metabolites. Finally, the purification procedure of CA III extraction was examined with respect to enzyme homogeneity.

Using the pH-stat assay system, experimental results indicated that CA III isoenzymes purified from chicken and human skeletal muscle exhibit an enhanced bicarbonate dehydration activity when phosphate is present, under steady-state conditions. The concentration range over which these effects are observed (5-50 mM) corresponds to the normal range of inorganic phosphate detected in whole muscle during exercise (4-25 mM) (Bevington et al, 1986). The levels of P_i detected in whole muscle may not entirely reflect the concentration in specific fibre types, but it is generally assumed that many skeletal muscles in higher organisms are a mix of fibre types. Thus, it is conceivable that CA III could be exposed to transitory high levels of inorganic phosphate.

The location of CA III in type I muscle fibres under precise hormonal and neuronal control is one of the fascinating enigmas of carbonic anhydrase biochemistry. Whether or not tissue specificity is linked to phosphate metabolism in muscle physiology poses an interesting question. Certainly free inorganic phosphate availability

and transport must play an integral role in the ability of slow-twitch muscle to sustain prolonged work. Continued contraction in these muscles is dependent on oxidative phosphorylation fuelled by glucose and fatty acids, following depletion of stored phosphocreatine.

Hudlicka examined the leakage of phosphate from rat soleus and gastrocnemius muscles and reported that 600% more phosphate was released from fast muscle as compared to slow muscle. The author postulated that the leakage of phosphate could be a causative factor in fatigue. It could be argued that in type I fibres localised phosphate pools are more likely to occur than in muscles containing a high proportion of type IIb fibres.

Experiments were conducted which essentially mirrored the possible physiological exposure of CA III to fluctuating high and low levels of P_i . It appears that chicken CA III responds instantaneously to P_i concentrations of 25 mM or more with subsequent enhancement of bicarbonate dehydration activity. Moreover, transfer of the P_i -modified enzyme to a low $[P_i]$ medium, results in enhanced activity for up to twenty minutes, before complete phosphate equilibration with the surrounding environment occurs. Consequently, slow dissociation could sustain the P_i effect under conditions of fluctuating phosphate concentration.

The potential for prolonged muscle contraction depends on the mobilisation of stored endogenous fuels and maintenance of ATP levels, with minimal perturbation of charge, osmotic balance or end product effects on pH. In addition, substrate flux must be balanced with O_2 flux (Hochachka, 1985). The efficiency of this process is therefore dependent on the rephosphorylation of ADP and the electrogenic ATP-ADP exchange in the mitochondria, the conversion of AMP to ATP by

adenylate kinase, the mobilisation of fuels particularly fatty acids and ultimately the pH (Jong and Davis, 1982; Connett et al, 1982). In contrast to a previous report on bovine CA III (Engberg et al, 1985), the results presented here indicate that chicken CA III is responsive to changes in pH between 6.7-7.5. This response is heightened in the presence of phosphate. Consequently, a combination of high $[P_i]$ and low pH results in a 400% increase in HCO_3^- dehydration activity.

Assuming that CA III is concerned with acid-base balance, it could be argued that during exercise when $[P_i]$ increases and pH decreases, CA III can rapidly respond to its changing environment by the interconversion of CO_2 to HCO_3^- and H^+ . CO_2 is thus rapidly transferred to the cell interphase for diffusion into the plasma.

Bicarbonate ions are not freely permeable to cell or sarcolemmal membranes. Recovery of acid-base balance status in the aerobic muscle after exercise probably involves the efflux of acid equivalents in exchange for sodium ions and the diffusion of CO_2 . The chloride/ HCO_3^- co-transport system is responsible for the inward transport of acid equivalents when the pH rises. Both systems are regulated by intracellular calcium and indirectly by inorganic phosphate levels. Several authors have presented evidence for a sarcolemmal CA and Geers et al (1985) have suggested that CA associated with the sarcoplasmic reticulum provides a rapid proton sink for the immediate exchange of H^+ with Ca^{++} . Calcium sequestration and mobilisation, a necessary prerequisite for muscle contraction, is an extremely rapid process and counter ion movements involving Mg^{++} and K^+ are thought to be too slow to account for the release of Ca^{++} during contraction.

Recently, Wetzel and Gros (1990), reported that there are two distinct sarcolemmal carbonic anhydrases in white and red muscle, with the former exhibiting twice the activity of the latter. Interestingly, sarcolemmal carbonic anhydrases are catalytically active in the interstitial space rather than the intercellular space. The extracellular buffering system for aerobic muscle is mainly concerned with H^+ uptake in interstitial fluid and plasma, which accelerates CO_2 uptake by the blood, whereas the anaerobic system includes efflux of lactate as well as H^+ from the active muscle cell. The presence of two distinct membrane-bound carbonic anhydrases may be related to the cytosolic location of CA III in red fibres and CA II in white fibres. Maximal efficiency would be achieved by the co-operative functioning of the cytosolic carbonic anhydrases, the membrane-bound enzymes and the transport systems.

An immunochemical survey of human colonic tissue and colonic neoplastic tissue showed that whilst sodium-potassium stimulated ATP-ase was localised at the membranes of both tissues, CA I and CA II were only found in the normal sections (Gramlich *et al*, 1990). In normal kidney, CA IV accounts for only 5% of the total activity of CA, yet it plays a major role in HCO_3^- reabsorption in the proximal tubule (Sato *et al*, 1990). Isoenzyme co-operation and linked regulation of transport systems could be under hormonal control. Correlations have been observed between duodenal CA and $Mg^{++}-HCO_3^-$ ATPase activities and serum levels of thyroxine (T_4) (Suzuki *et al*, 1989). Fibre type composition is directly related to thyroid hormone levels and it would be interesting to monitor the relative proportions of the sarcolemmal red and white muscle carbonic anhydrases after thyroidectomy. Arguably, CA III with its low activity is an

unsuitable isoenzyme to maintain pH balance, yet its high concentration and conserved sequence signifies a distinct tissue-specific function and a possible co-operative role with other systems. The results in this thesis suggest that phosphate decreases the K_M towards HCO_3^- ; hence in its peculiar environment CA III may be more effective than had hitherto been realised.

It is pertinent to question whether experiments conducted at 2°C in a cell-free system can be equated to an integrated complex homeostatic system. No studies have been conducted on the temperature dependence of CA III and excessively high rates preclude measurements above 2°C using the pH-stat assay system. Theoretically any response at 2°C may be more pronounced at physiological temperatures.

In addition to P_i , a number of other phosphorylated metabolites and anions increased the bicarbonate dehydration activity of chicken and human CA III, namely ATP, ADP, BPG, 3-PG, pyrophosphate and hydrogen sulphite. Notably, AMP and phosphocreatine were without effect. It is difficult to sterically relate this varied group of activators, yet activation by any one of these metabolites or ions effectively precluded further activation by another, suggesting a common feature of the mode of action. No phosphatase activity by CA III was demonstrated with any of the activators.

The unique invariant basic residues in CA III isoenzymes at positions 64, 67 and 91 have been the focus of intense investigation and speculation. His-64 in CA II is implicated in the rate-limiting proton shuttle the reaction mechanism, and the substitution of arginine or lysine in CA III is attributed to its low activity. However, CA II mutants with acidic or basic residues at 64 exhibit no change in activity (Forsman et al, 1988). Interestingly, in an

unbuffered medium, replacing His-64 by Ala-64 in CA II decreases the rate of proton transfer by 20-fold (Tu and Silverman, 1988). The studies in this laboratory clearly underline the profound effect of ions on CA III activity and emphasise that these responses are peculiar to particular buffer systems' effects. Borate, barbitol, mops, pipes and hepes buffers have no effect on activity measurements. In contrast, hydrogen sulphite activates chicken CA III by 350% and inhibition by Cl^- ions is effectively counteracted by the inclusion of P_i . It would appear that these activating ions and metabolites enhance activity by ionic interaction with CA III rather than mediating their effect by simply participating from the external medium.

It is worth noting the bulky nature of some of the activators mentioned above. $\text{CO}_2/\text{HCO}_3^-$, attached to the Zn moiety, resides in a deep water filled pocket at the active site cavity (Merz, 1990). Bearing in mind the large phenylalanine side chain at position 198 of CA III, it is quite possible that steric hindrance would prevent a large ATP molecule, for instance, from binding deep within the cavity. Lys 64/Arg 64 and Arg 69, situated at the mouth of the cavity would seem ideal residues for interaction with bulky metabolites. However, studies on the proton transfer in catalysis of human CA III by Tu et al (1990), indicated that the size of the molecule comprising the buffer was more important than the presence of basic residues at positions 64 and 69.

Catalysis of carbonic anhydrase can be effectively divided into two separate processes, the interconversion of CO_2 and HCO_3^- :-

$$\text{HCO}_3^- + \text{E}2\text{nH}_2\text{O} \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O} + \text{E}2\text{nOH}^-$$
and the regeneration of zinc bound water or hydroxide ion by the transfer or

removal of a proton:- $E2nOH^- + BH^+ \rightleftharpoons E2nH_2O + B$. Thus, bicarbonate dehydration depends on the donation of a proton from the buffer to zinc-bound hydroxide, and carbon dioxide hydration by the transfer of a proton from the Zn-complex to a residue then to the medium or directly to the buffer. In CA I and II, His-64 is thought to facilitate this proton transfer, whereas in CA III proton transfer is direct to the medium. Tu and Lindskog (1990) investigated these two mechanisms in CA III and reported that certain small buffers facilitated the proton transfer step without effecting the CO_2/HCO_3^- interconversion. Moreover, replacement of the bulky Arg-64 residue with an uncharged Asn residue in human CA III permitted a more efficient proton transfer from imidazolium cations to the zinc hydroxide.

Thus, arginine hinders this process. If phosphate and phosphorylated metabolites mediate their enhancing effects by a corresponding mechanism, it is difficult to visualise that these bulky interactions are of selective advantage, unless the ionic interaction of phosphate or phosphorylated metabolites results in a conformational change which promotes proton transfer.

Previous studies in this laboratory with the arginine-modifying agent 2,3-butanedione indicate that this activating agent combines with active site arginine or lysine residues and, after an initial activating effect on HCO_3^- dehydration activity, produces a slow conformational change in CA III over a twenty-four hour period. The results in this thesis suggest that BD-modification and P_i -modification of chicken CA III may share common features, since prior treatment of chicken CA III with BD precludes further modification by pretreatment with P_i , as determined by enhanced

HCO_3^- activity. Yet both BD-modified and P_i -modified enzyme can be additionally activated by augmenting the medium with phosphate. Research on the formation and dissociation of the P_i -chicken CA III interaction strongly indicates that a conformational change ensues after P_i interaction. It could be postulated that binding of phosphate related ions or phosphorylated metabolites is aided by the basic active site residues and that this interaction leads to a conformational change and a more efficient proton transfer.

A simple buffer effect deep within the active site cleft with no ionic interaction seems unlikely, it would certainly be imprecise and with no regulatory control. Of course, the CO_2 and HCO_3^- interconversion of CA III may be irrelevant in the muscle cell, and the 'true' substrate may be awaiting discovery!

Recent developments in the field of protein regulation stimulate the suggestion that, CA III may possess certain of the requirements for targeting by a regulatory interacting protein, such as an $\text{ATP}/\text{P}_i/\text{PP}_i$ binding site, active site Lys/Arg residues and unique tissue specificity. Ubiquitin, for instance, regulates H2 histones by lysine interaction with the Arg-Gly-Gly COOH-terminal sequence, and similarly targets cytosolic proteins, for subsequent proteolysis, via an ATP intermediate (Wilkerion and Audhya, 1981, Haas *et al*, 1983; Ziegenhager *et al*, 1990). It would be interesting to examine the effect of inositol triphosphate on the HCO_3^- dehydration activity.

The probable link between phosphate, basic residues in the active site and enhancement of HCO_3^- dehydration has been discussed at length. Is there any evidence for the involvement of other active site residues? Certainly, competitive experiments with P_i -modified and BD-modified chicken CA III suggest that whilst these

two interactions may encompass similar processes, phosphate probably has an additional effect that does not involve Arg/Lys binding. Furthermore, HCO_3^- dehydration of human CA I is enhanced by phosphate, yet this isoenzyme possesses no active site basic residues and has histidine at position 64. Isoenzyme I-specific catalytic and inhibition binding properties are thought to reside partially in the invariant histidine residue at position 200. Bahravan et al (1990) investigated the properties of a human CA II thr-200 ----> his-200 mutant and concluded that his-200 was implicated in isoenzyme I characteristics but was not the only determinant. Position 200 is occupied by threonine in CA III, so this residue can be effectively discounted in phosphate interaction in CA I.

CA I additionally responds to mops buffer with an enhancement of HCO_3^- dehydration activity, in contrast to CA III. This large molecule may be accommodated more readily in the wider cleft of CA I, implying that activation of HCO_3^- dehydration may be a property of access rather than specific binding sites. The sulphonamide inhibitor, acetazolamide binds at positions 91, 121 and 131 as well as interaction with the zinc-hydrogen bond network. BD-modification dramatically reduces the I_{50} for acetazolamide of human CA III, whereas the effect of P_i on the I_{50} is slight. Competition in binding at Arg-91 between P_i and acetazolamide may be of minimal significance; conversely BD interaction at Arg-91 appears to interfere with sulphonamide bonding.

Engberg and Lindskog (1985) investigated the effects of varying alkylating agents on the properties of bovine CA III. They concluded that modification of Cys-183 and Cys-188 occurred most readily, resulting in enhanced CO_2 hydration activity followed by

derivatization of the other thiol groups Cys-66, Cys-203 and/or Cys-206. No evidence of blocked cysteine residues in purified CA III samples is available, although glutathione linkage has been demonstrated in tiger shark red-cell CA. Whatever the mechanism or mechanisms of activation by BD, P_i or thiol modifying agents, it seems likely that interaction may involve specific binding with consequent conformational changes. 25

Detailed kinetic studies of the HCO_3^- dehydration activity proved problematic owing to the high K_M of CA III towards HCO_3^- , and inaccuracies were attributed to limitations of the pH-stat assay system at high substrate concentrations. Bahravan *et al* (1990) commented on reports, that under certain conditions, CA I displayed non-Michaelis-Menten behaviour due to the slow rate of the buffer dependent step. Additionally, Ren *et al* (1988) reported that the kinetic patterns of HCO_3^- dehydration of bovine CA III suggested a build up of the E-Zn-H₂O species at steady state and concomitant non-Michaelis-Menten kinetics. If the low activity of CA III is ascribed to the proton transfer step, then buffer/enzyme interactions in the reaction vessel could contribute to inconsistent data. Certainly, the N₂ flow rate through the reaction vessel and hence the degree of agitation produces a marked response on activity measurements. Possibly, with increasing substrate concentrations from a wide range of 30-350 mM, the N₂ rate could be a limiting factor and produce anomalous results. Notwithstanding the intricacies of kinetic measurements, measurements at 30 mM NaHCO₃ were consistent and reliable, and similar effects were monitored on many separate occasions.

One of the salient features of the carbonic anhydrase molecule is the deep active site cleft which encompasses a considerable proportion of the small molecule. As a consequence, it is surprising that many authors report that the location of the active site for the CO_2 hydration activity is separate from the 4-NPA esterase site. The results in this thesis indicate that this property is also shared by chicken CA III, since phosphate had no effect on the K_M or k_{cat} for 4-NPA hydrolysis. Arginine modification by BD, however, results in an increase in k_{cat} but no change in K_M for ester hydrolysis. The affinity of CA III for 4-NPA is unaffected by BD-modification, whereas the arginine modification by BD alters the catalytic process. The BD molecule is covalently linked to arginine residues, and the BD-arginine complex stabilised by borate. By some undetermined mechanism the BD-arginine complex effects the catalytic processes of both HCO_3^- dehydration and 4-NPA hydrolysis without interfering with 4-NPA binding. A surprising observation in view of the size of the substrate molecule unless the BD-borate spans both sites.

It is worth noting that the 4-NPA activity is extremely low in chicken CA III, indeed it has only 0.1% of the activity of human CA II, as determined under steady-state conditions. A comprehensive investigation of the purification procedure revealed the presence of one particular contaminating factor. This protein, a dimer, of approximately 70 kD, exhibited 4-NPA esterase activity which was insufficient to account for CA III esterase activity in a 95% pure sample. Another unexpected property of this contaminating protein was most revealing since it possesses phospho-monoesterase activity, and questions the claims that CA III has this unique property. A measured acid phosphatase activity was also observed in human CA I.

The levels of absorbance change in all the phosphatase activity experiments were exceedingly small, and many pure preparations exhibited no activity at all. It is a tenable hypothesis, that since CA III extraction procedures were fairly uniform, this acid phosphatase activity is not a feature of CA III or indeed any of the carbonic anhydrases, but a property of this contaminating protein.

It became apparent that this molecule separated with CA III either because of intrinsic comparable physical properties or it was complexed with CA III. All activity and other separation investigations excluded a protein-CA III complex in vitro. However, it emerged that this dimeric basic protein responded or interacted strongly with sulphonamide. Many physiological studies using whole muscle are based solely on sulphonamide inhibition measurements, and in some instances relative inhibition constants are related to particular isoenzymes. Apparently, red skeletal muscle contains a cytosolic protein of unknown function and in considerable concentration which responds to sulphonamide and could possibly undermine these measurements.

The investigation of the function of this protein is just one of the many potential directions which have emerged during this research on muscle carbonic anhydrase III. I feel convinced that this interesting, oft frustrating molecule will reveal many more of its secrets as technology develops. Time will tell if any of the information in this text contributes to the continuing saga of muscle carbonic anhydrase . . .

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